STUDIES ON THE IMMUNOCHEMISTRY AND SEROLOGY OF *TAENIA SAGINATA* INFECTION IN CATTLE

by

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PREFACE

The work described in this thesis is original and has not been submitted in any form to any other University. It was carried out by the author in the Centre for Tropical Veterinary Medicine, University of Edinburgh, under the supervision of Dr. M.M.H. Sewell and Dr. G.J. Gallie.

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SUMMARY

The work reported in this thesis fell into three main sections. The first was a study of the immunochemistry of a saline extract of T. saginata proglottids (SE) with particular reference to the antigens involved in the serological response of cattle to infection with T. saginata. It was hoped that this information would facilitate the production of relatively pure and specific T. saginata antigens for use in serodiagnostic tests for this infection.

The second section was concerned with an investigation of the potential of certain serological techniques, not previously used with T. saginata infections in cattle, for use in serological investigations of experimental and field infections. In the third and final section these serological techniques were used to measure the serum antibody levels in cattle both experimentally and naturally infected with T. saginata. Where possible, partially purified saline extracts of T. saginata were used as antigens in these techniques.

The immunochemistry of SE was studied by gel filtration on Sephadex G200, Sepharose 6B and Sepharose 4B, by ion exchange chromatography and by immunoadsorption techniques. It was found that SE consisted of two main groups of antigenic molecules. The first group were of a molecular weight equivalent to that of the macroglobulin component of bovine serum and contained most of the haemagglutinin activity. The second group were of a molecular weight

equivalent to that of the globulin component of bovine serum and contained mainly gel precipitin activity. The immuno-adsorption studies on this extract showed the method to be potentially valuable for the purification of these helminth antigens but much work still remains to be done on this relatively new approach.

The micro immuno-precipitation (MGP) and the indirect haemagglutination (IDH) techniques had already been used in bovine cysticercosis research prior to the commencement of this work and only minor modifications have been made to these procedures. However, the haemagglutination inhibition (HI), enzyme-linked immunosorbent assay (ELISA) and the soluble antigen fluorescent antibody (SAFA) techniques have each been adapted to the study of the T. saginata system in cattle for the first time. A comparison of SAFA and ELISA showed that ELISA was the preferable of the two techniques for experimental work. The technique also has potential as a versatile and sensitive tool for studies on naturally infected cattle. The HI technique proved effective in detecting haemagglutinin activity in fractions of SE produced by column chromatography.

Studies on cattle experimentally infected with T. saginata showed that the ELISA technique compared favourably with the IDH and MGP techniques. ELISA could in fact be used to detect an antibody response to both haemagglutinin and gel precipitin antigens. In contrast the IDH technique mainly detected an antibody response to haemagglutinins and the MGP technique an antibody response to gel precipitin antigens.

A limited study on cattle naturally infected with *T. saginata* showed, however, that ELISA is not presently a reliable test for *T. saginata* infection in the field. In particular, further work requires to be done on the antigens used in serological techniques, although there was an indication that some, but not all, cross reacting antigens could be removed by gel filtration and immunoadsorption.

CHAPTER 1

1. General Introduction and Literature Review.

1.1. General introduction.

The cestode Taenia saginata Goeze, 1782 is a parasite of world wide human and veterinary interest (Froyd, 1965 and the WHO/FAO/CIE Animal Health Yearbooks), particularly in areas where beef production plays an important role in the local economy. In regions of high prevalence, such as East Africa, the improvement and development of cattle rearing and international trading in beef is hindered because so many beef carcases are condemned at slaughter due to infection with T. saginata cysticerci. In other areas of the world sporadic infections in beef cattle can result in large financial losses to individual farmers. Even in countries with a low incidence the total annual loss to the beef industry may be considerable.

Current control measures for this parasite are relatively ineffective because lightly infected carcases, which are epidemiologically important, are difficult to detect, either by present meat inspection or serological techniques. The main drawback to serodiagnosis of this infection in cattle or man has been the lack of sensitivity and specificity of the techniques combined with a high level of cross reactivity with other cestode species, resulting in false positive and false negative results.

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1.2. Literature review.

1.2.1. The life cycle.

The life cycle of T. saginata is well known and is dependent on the association of man, the only known definitive host for the adult tapeworm, with domestic cattle the main intermediate host. Following the suggestion of Pawlowski & Schultz (1972) and to conform with other zoological nomenclature, the term T. saginata will be used for all stages of the life cycle.

The adult parasite is usually found in the intestines of the definitive host, from where the proglottids containing mature eggs are voided with the faeces. Cestode larvae are often less host specific than the adult parasites and there have been several reports in the literature of T. saginata cysticerci in various species of ruminants. However, the main intermediate hosts responsible for transmission to man are undoubtably domestic cattle. Cattle become infected when they ingest the eggs. The oncospheres hatch in the intestinal tract, burrow through the walls of the intestine and are carried around the body either by the blood or the lymph. Prenatal infection in cattle can occur but may be relatively uncommon (Urquhart, 1961). However, in the period from 1959 - 1971, 2.7 per cent of Kenyan calves inspected at up to 1 week old were found to be infected with T. saginata cysticerci (Nganga, 1974). Developed cysticerci (often referred to as Cysticercus bovis) are most often found in the musculature, but they can also be found in such sites as the lungs, kidneys and liver etc.

Man becomes infected when he eats raw or partially cooked

meat from animals infected with T. saginata cysticerci.

1.2.2. Medical and economic effects of T. saginata.

Medical complications can be caused by the adult tapeworm and an economic loss to agricultural communities is caused by the larvae in the cattle. T. saginata is not usually considered a serious medical problem in man, especially when compared to other parasitic diseases such as trypanosomiasis and schistosomiasis. Infection with the parasite is rarely fatal unless the tapeworm is sited in an unusual location. In most cases there are few or no ill effects resulting from infection, but symptoms most commonly cited are abdominal pain, nausea, weakness, increased appetite, headache etc. The parasite may also have a psychologically disturbing effect on some people. The medical cost of treating patients has been estimated to be between 8 per cent (Logan, 1967) and 5 per cent (Mielke, 1969) of the annual loss due to cysticercosis.

Considerable information exists about the financial losses due to T. saginata cysticercosis in cattle. These losses are due to such things as the outright condemnation of heavily infected carcases, downgrading the value of carcases with lighter infection and deep freezing carcases to kill the cysts. In the United Kingdom the annual loss due to downgrading carcases was estimated to be between £150,000 and £210,000 (Silverman, 1955). The yearly loss

in Eire was estimated to be £100,000 (Logan, 1967) and in West Germany 5 million DM (Friedrich, 1961). The annual loss to Kenya was estimated at 1 million U.S.A. Dollars (Mann, 1971) and if the cattle production target of 500,000 head per annum was met with no improvement in the infection rate, the loss to the beef industry would rise to £2.5 million per annum. This author, assuming a similar infection rate in the neighbouring countries of Tanzania, Uganda, Ethiopia and Burundi, estimated that the annual loss to all these countries would be about £10 million and this figure did not take into account the loss of high-price export markets. With losses such as these, it is hardly surprising that the development of a profitable beef industry in these countries has been hampered. It is therefore of considerable importance that the incidence of T. saginata cysticercosis should be reduced, particularly in areas such as East Africa.

1.2.3. Control of T. saginata.

In theory this parasite could be controlled at a number of stages in its life cycle but this has proved difficult in practice. One procedure would be mass diagnosis followed by compulsory treatment of persons infected with T. saginata, together with improved sanitation such as improved drainage and sewage treatment plants. Such schemes are however expensive and require social acceptance of control measures, which can only be achieved by educating the public. Almost every stage is beset by problems and is often staged against a background in which

little finance is available and in which other more pathogenic diseases, both human and veterinary, predominate. Where such control measures have been applied, as in the USSR, they have significantly reduced but not eliminated human taeniasis (Prokopenko, 1968). Similarly improvements in the sanitary and economic conditions in Israel have almost eliminated T. saginata (Witenberg, 1968).

Another stage in the life cycle which could be attacked is the eggs on the pasture. Given suitable conditions T. saginata eggs can survive for long periods on pasture. The main factor affecting the survival of taeniid eggs is desiccation (Laws, 1968) and both the amount and persistence of surface moisture on pasture are important. Temperature also plays a significant role. At 4-5°C the eggs can survive for at least 168 days (Froyd, 1962) but extremes of temperature on either side reduce survival time.

Taeniid eggs can survive most chemical disinfectants (Laws, 1967 and Mackie & Parnell, 1967). However, in vitro techniques were used in these studies to assess the viability of the eggs and the chemicals used may have affected the hatching solutions (Mackie & Parnell, 1967). "Satisfactory ovicidal effects" have been reported (Gall & Wikerhauser, 1969) with 0.5 per cent toxychloramide sodium, 10 per cent formalin or 2 per cent NaOH, but again in vitro techniques were used for assessing egg viability. In general this aspect of control has not been widely investigated and at present there is no proven ovicidal treatment for pasture contaminated with T. saginata eggs. To leave such pasture

ungrazed by cattle so that the eggs may die is a possibility but it would not be economically practical in many cases.

The other main point at which the parasite's life cycle can be attacked is by detection of the cysticerci in the cattle, either post-mortem or ante-mortem. Meat inspection is one of the foremost public health measures for the prevention of T. saginata transmission. However, meat inspection alone cannot be expected to eradicate T. saginata, especially as it is conducted at present. The procedure, although very efficient in detecting heavily infected carcases, is not very efficient in detecting light infections. Meat inspection in the U.S.A. failed to detect infection in 22 out of 80 cattle which, on later investigation by more careful dissection, were found to be lightly infected (Dewhirst, Cramer & Sheldon, 1967). There has also been some controversy about "predilection sites". Various authors have suggested that cysts have a tendency to develop in certain organs or muscles in the body. Sites mentioned have included the heart, masseter and shoulder muscles etc. Pawlowski & Schultz (1972) in their detailed review recommended that the choice of muscles should be based on studies done in each country or region because of possible strain differences in T. saginata found in different areas. Ultraviolet light has been reported to increase the probability of finding cysts in a carcase (Gibson, 1969) but the procedure has not been widely adopted probably because the number of cases detected by this method is little more than the detection rate using thorough

conventional examination.

Once T. saginata cysticerci have been found in a carcase several steps can be taken to prevent transmission of the parasite. The meat can be discarded, although this is not very practical except in cases of very heavy infection. A proposal to use gamma radiation to kill the cysts (Pawel & Janíečk, 1963 a & b) has not been used in practice. A more acceptable alternative is to boil or freeze the meat. The upper thermal death point of T. saginata is 56°C (Allen, 1945 and Allen, 1947) so heating meat to a temperature in excess of 56°C is effective provided the meat is heated all the way through. Unfortunately this process is costly and diminishes the potential value of the meat. It also requires large scale canning facilities if the procedure is to be carried out routinely and the meat stored for any time. Freezing meat at -10°C for 10 days effectively kills cysts (Hajduk, Muller, Saalbreiter, Eymmer, Hiepe, Bruckner & Wilhelm, 1969). However, this procedure is expensive and causes the quality of the meat to deteriorate. At present, therefore, freezing cannot be used routinely unless cysts have been demonstrated in the carcase.

Chemotherapy is potentially an attractive way of killing cysts in live cattle, but despite trials with several types of drug none have so far proved effective, although a recent report has suggested that Praziquantel (Bayer) has such activity (Thomas, Ginnert, Pohlhe & Seubert, 1975).

As an alternative to or an adjunct to post-mortem diagnosis, attention has been directed towards ante-mortem diagnosis of infection mainly involving serological

techniques but, for reasons which will be outlined (1.2.6.7.), this has not so far proved effective. There remains the problem of detecting the low levels of T. saginata infection in cattle which often escape visual meat inspection methods in both the abattoir and by consumers and which are, therefore, epidemiologically very important.

1.2.4. Progress towards immunizing cattle against T. saginata.

Various procedures have been tried aimed at producing immunity in cattle to T. saginata infection. The original suggestion (Penfold, 1937) of giving cattle T. saginata eggs orally, for the reasons outlined later (1.2.5.), would only be of use in protecting older cattle not previously infected and would temporarily result in the presence of viable T. saginata cysticerci in the musculature. An alternative method, per os infection of cattle with irradiated eggs (Urquhart, McIntyre, Mulligan, Jarrett and Sharp, 1963 and Urquhart, 1966), does not result in solid immunity to challenge infection with T. saginata eggs. Intramuscular injection of artificially hatched and activated T. saginata eggs has the disadvantage that some of the cysts may be disseminated throughout the carcase, nor does the technique always produce a solid immunity, (Wikerhauser, Žukovic & Džakula, 1971 and Sewell & Gallie, 1974). Neither does intramuscular injection of calves with artificially hatched and activated Taenia hydatigena eggs (Wikerhauser et al, 1971). Intramuscular injection with a somatic antigen

has been reported to have produced resistance to re-infection in one trial in six-month old cattle (Sewell & Gallie, 1974) but an attempt to repeat the experiment proved only partially successful, (Gallie & Sewell, 1976, pers. comm.).

Although all the above techniques are potentially of use in immunising previously uninfected older cattle, none of the methods are known to be capable of producing immunity in new-born calves. These young cattle are immunologically unresponsive to T. saginata infection (Soulsby, 1963). The successful use of immunological means to control this parasite would necessitate protecting calves from infection until they are old enough to be properly immunised or finding a way to break down the tolerance to the neonatally or even pre-natally acquired cysticerci in the musculature. Unfortunately Froyd (1964 a) failed to produce passive immunity in calves.

1.2.5. The relationship between T. saginata and its intermediate host.

Cattle aged about 2½ years develop a strong resistance to reinfection with T. saginata following a single large oral infection with T. saginata eggs (Penfold, Penfold & Phillips, 1936 and Penfold & Penfold, 1937). The relationship between T. saginata and its intermediate host is complicated by the fact that young cattle orally infected with T. saginata eggs within the first week of life do not develop a strong serological response or resistance to later challenge (Soulsby, 1963 and Gallie & Sewell, 1974a). Cattle

given an oral infection with T. saginata eggs at 3 months of age are only partially resistant to challenge infection when 9 months old (Gallie & Sewell, 1974b), although these calves displayed a relatively strong serological response as measured by the indirect haemagglutination technique. Cattle given a single oral primary infection at 4-6 months of age are completely resistant to challenge infection at 10 months of age but they can be reinfected at 20 months of age. These cattle developed a relatively strong serological response as measured by the complement fixation reaction (Soulsby, 1963).

In countries where the prevalence of T. saginata is high, naturally infected calves can rarely be reinfected. In Kenya calves over 4 months old have a high degree of resistance to reinfection with T. saginata which becomes complete at an age of about 1 year (Peel, 1953; Froyd, 1960; Urquhart 1961; Froyd, 1964 b & c and Graber & Throme, 1964). In spite of this, in countries where infection is widespread both viable and dead cysts are frequently observed in cattle over 1 year of age at slaughter. These cysts are considered to have survived from infections acquired early in life before the host had developed resistance (Urquhart, 1961 and Froyd, 1964 b & c).

Resistance to reinfection of cattle with T. saginata is acquired as a result of infection with T. saginata and not from age per se, since previously uninfected cattle of all ages are susceptible to infection with T. saginata (Vegors & Lucker, 1971).

Under East African conditions calves probably become resistant and maintain their resistance because of constant stimulation arising from frequent ingestion of T. saginata eggs (Gallie & Sewell, 1972). Calves orally infected at 2-3 days of age and then given small weekly doses of T. saginata eggs are resistant to challenge infection at 12 months old, whereas similar calves given only the single infection at 2-3 days of age are susceptible to reinfection 12 months later (Gallie & Sewell, 1974a). Both groups of calves still contained viable cysts, considered to have probably come from the initial oral infection. The serological response of the serially dosed group did not become significantly stronger than that of the single dose group, although the former was resistant to challenge infection and the latter was not. This experiment supports the hypothesis based on field observations in Kenya (Urquhart, 1961 and Froyd, 1964 b & c) that cattle which are resistant to reinfection can continue to harbour viable cysts from earlier infections without displaying a strong serological response.

1.2.6. The serology of T. saginata infection in cattle.

Immunodiagnosis is used, with varying degrees of success in a wide number of parasitic infections and this has been the subject of review (Fife, 1971 and Kagan, 1974). There are many publications on the serological response of cattle to infection with T. saginata. Serological techniques have been used to monitor the response of cattle to

experimental infection and to investigate the potential of some techniques for ante-mortem diagnosis in cattle. The following review does not attempt to list all the publications but merely to indicate the various tests used and their relative merits and disadvantages. The methods of antigen preparation are also mentioned since a full comparison of serological techniques cannot be made unless this is taken into consideration.

Other parameters associated with infection such as serum globulin levels have also been investigated.

1.2.6.1. The intradermal (ID) or skin test.

The ID test detected 83·1 per cent of cattle infected with T. saginata cysticerci, but 11·2 per cent false positive reactions occurred with Fasciola hepatica and Dicrocoelium dendriticum (Bugyaki, 1961). Cyst fluid or aqueous extracts from T. saginata or other helminths were used as antigens.

Again in East Africa, several means of antigen extraction were tried to obtain polysaccaride and carbohydrate fractions from lyophilised and delipidised samples of T. saginata cysticerci, T. saginata strobilae, F. hepatica, Echinococcus granulosus and Taenia taeniaeformis cysticerci (Froyd, 1963). None of these extracts were specific for the diagnosis of T. saginata cysticercosis by the ID test. The methods used in their preparation were listed to save other workers covering the same ground in a search for a specific antigen for an ID test

for bovine cysticercosis. The ID test only detected 24 out of 42 cases of infected cattle and gave 34 false positive reactions (Kosminkov, 1965). An extract of fresh lyophilised T. saginata scolices, which had previously been fixed in 96 per cent alcohol, was used as antigen. Results with polysaccaride and acid soluble fractions were worse. However, in the U.S.A., the ID test was better at detecting infected cattle than conventional meat inspection techniques, although worse than the indirect haemagglutination (IDH) test (Dewhirst, et al 1967). A glycine buffered saline extract of lyophilised and delipidised T. saginata strobilae, prepared according to Chaffee, Bauman & Shapilo (1954) was used. After aqueous and saline extraction, ammonium sulphate precipitation and gel filtration chromatography of the antigen, the specificity of the ID test was improved (Shekhovtsov, Pinchuk & Bidenko, 1972).

The response of cattle to experimental infection with T. saginata eggs was monitored using the ID test, but the results were not specific. The test first gave a positive reaction 21 days post infection (pi), remaining so for up to 94 days although there was still a slight reaction 11 months later (Shoop & Lamina, 1970).

The main advantage of the ID test is its simplicity but it is not quantitative and the cattle need to be handled at least twice. The only criterion for determining a positive result is by weal size and this can cause problems as it is totally arbritary (Dewhirst et al, 1967). The test

also lacks specificity and sensitivity.

1.2.6.2. The complement fixation (CF) test.

In a test on 2021 slaughter cattle of which 8 per cent. had T. saginata cysticercosis, 62 per cent of the cases were detected by the CF test but there were 15 per cent false positive results (Frick & Süsse, 1970). A delipidised saline extract of T. saginata proglottids was used as antigen. These results compared with a 42 per cent detection rate for the IDH test which gave 8 per cent false positive results. The ID test and latex agglutination (LA) test gave poorer results.

In another trial sera from cattle shown by normal meat inspection procedures to contain T. saginata cysticerci (either living or calcified) were compared with those considered to be free from cysticerci (Süsse & Frick, 1970). Positive results with the CF test were given by 79 from 120 infected carcases and 20 from 120 apparently uninfected carcases. In comparison 22 from 48 cattle showing cysticerci and only 4 from 48 cattle not showing cysticerci gave positive reactions with the IDH test.

However, in Chad the diagnosis of T. saginata cysticercosis by the CF test was complicated by low titres and in some cases by cross reactions with Fasciola gigantica and Monezia expansa, (Martin, 1972). The LA and IDH tests were more satisfactory than either the gel-precipitation (GP) or the CF test, which gave low titres in 80 per cent of the infected cattle. A delipidised aqueous extract of

T. saginata was used as antigen.

When the CF test was used to monitor the response of 4-6 month old calves to experimental oral infection with T. saginata eggs, the antibody levels showed two peaks (Soulsby, 1963). The first was 4-6 weeks and the second 6-9 months pi. In contrast calves infected at birth showed a very poor antibody response. An extract of lyophilised T. saginata cysticerci was used as antigen.

In another experimental infection (Lamina & Hein, 1970) the CF test was found to be effective over a period of 2-3 months pi. The serological response was similar to that reported by Soulsby (1963). However, after the 10th month pi antibodies could no longer be detected in the serum, although living T. saginata cysticerci were still in the musculature. An aqueous extract of T. saginata proglottids was used as antigen.

The CF test is relatively complex and does not give any indication of the number of antigen/antibody complexes involved in a serological response. The main advantage of the test is its sensitivity when compared with other techniques such as the IDH, LA and ID tests. This sensitivity however, is of little use when a large number of cross reactions with other helminths may occur.

1.2.6.3. The latex agglutination (LA) test.

The LA test has been reported to be very specific compared with other serological techniques in detecting cattle naturally infected with T. saginata cysticerci.

Thus 32 out of 35 infected cattle were detected by the LA test and, although the test also gave positive results in 127 of 319 uninfected animals, the titres were much lower (Leikina, Sokolovskaya, Poletaeva, Astakhova & Moskvin, 1966). An extract of lyophilised T. saginata cyst scolices was used as antigen.

When the LA and IDH tests were used to monitor the response of cattle experimentally infected with T. saginata eggs at 4-5 months of age, a positive response was first recorded 10 - 40 days pi. The titre rose continuously until 120-180 days pi and was still high at 200 days pi, (Sokolovskaya, 1966). The antigen used for the LA test was a phosphate buffer pH 5·85 extract of lyophilised whole T. saginata cysticerci and the antigen for the IDH test was a phosphate buffer pH 7·2 extract of lyophilised T. saginata cysticerci.

A buffered saline extract of lyophilised T. saginata cysticerci was better than either a homogenate of T. saginata cysticerci, lyophilised T. saginata strobilae or a homogenate of T. saginata strobilae in detecting T. saginata cysticerci in slaughter cattle with the LA test (Sokolovskaya & Moskvin, 1967). These antigen extracts detected 98·7, 94·7, 82·0 and 58·5 per cent respectively of infected cattle but false positive reactions occurred with F. hepatica and hydatid fluid.

The LA test gave positive reactions with 109 of 113 slaughter cattle shown to be infected with T. saginata cysticerci, while in an experimental infection a positive

result was first found 2-6 weeks pi (Grossklaus & Walther, 1970). A delipidised aqueous extract of T. saginata proglottids was used as antigen. In a trial on 825 slaughter cattle (Fillipov, 1971), the LA test was positive in all 17 of the cattle which were found to be infected with T. saginata cysticerci by routine meat inspection techniques. Cross reactions occurred with cattle infected with F. hepatica, but not with other helminth infections or tuberculosis. An aqueous extract prepared from the scolices of T. saginata cysticerci was used as antigen.

In another trial on 2211 slaughter cattle (Kosminkov & Fillipov, 1971) the LA test was positive in 4.3 per cent of the cattle, but T. saginata cysticerci were found in only 2 per cent of the cattle by normal meat inspection techniques. Two false negatives were recorded, one calf had one cyst and the other two cysts in the heart. However, failure to detect cysts at meat inspection does not necessarily indicate that the cattle were not infected.

The LA test was positive in 39 out of 42 abbatoir cattle infected with T. saginata cysticerci alone and in 55 of 60 cattle infected with T. saginata cysticerci, hydatid and F. hepatica, (Alferova, Isankalova & Aslamov, 1972). In another group infected with F. hepatica and T. saginata cysticerci 100 out of 219 calves gave positive results as did 57 out of 135 cattle which were shown to be free of T. saginata cysticerci by normal meat inspection techniques. However, this does not necessarily indicate that these cattle were not infected with T. saginata cysticerci. Data

was not given on cross reactions in cattle infected with F. hepatica and/or hydatid but no T. saginata cysticerci.

1.2.6.4. The indirect haemagglutination (IDH) test.

The IDH test was found to be more sensitive than either conventional meat inspection techniques or the ID test in detecting infections of T. saginata cysticerci in cattle in the U.S.A. (Dewhirst et al, 1967). Cattle given an experimental oral infection with T. saginata eggs at 4-5 months old first gave a positive result with the IDH test at one month pi (Leikina & Ballad, 1970). The antibody titre reached a peak at 4-5 months pi and thereafter declined. The antigen used was prepared from T. saginata strobilae and cysticerci. In another experimental infection (Walther & Grossklaus, 1972) a positive result was first noted with the IDH test in the third week pi, but some of the animals did not give a positive reaction until the ninth week pi. The highest antibody titres observed were between 1:80 and 1:20,480. A delipidised phosphate buffered saline extract of lyophilised T. saginata proglottids was used as antigen. Sera from cattle orally infected with 100,000 T. saginata eggs at 3 months of age first gave a positive result with the IDH test 4-5 weeks pi (Gallie & Sewell, 1974b). Maximum antibody titres were between 1:128 and 1:4,096 and these remained high up to 44 weeks pi, before starting to decline. A borate buffered saline extract of mixed gravid and non-gravid T. saginata proglottids was used as antigen.

Calves infected orally from birth with T. saginata eggs did not give such a marked antibody response (Gallie & Sewell, 1974a).

Antigens prepared from Taenia crassiceps cysticerci and T. saginata cysticerci were shown to have equally strong sensitivity, but extracts from T. saginata strobilae gave the highest serum titres, (Hungerer, Henning, Enders & Zwisler, 1974) in the IDH test for T. saginata cysticerci. Saline extracts, delipidised saline extracts and ammonium sulphate fractionated extracts of delipidised material from T. crassiceps cysticerci and T. saginata cysticerci were prepared for comparison. Antigens prepared from T. crassiceps cysticerci or T. saginata cysticerci and strobilae were equally suitable for use in the IDH test for T. saginata infection in cattle (Henning, Hungerer, Enders, Zwisler, 1974), but the test could not distinguish the serum of normal cattle from that from cattle with less than 10 T. saginata cysticerci present.

1.2.6.5. The immunoprecipitation test or gel precipitation (GP) test.

Although the GP test lacks sensitivity as compared with other serological tests, it has the advantage that it gives some indication of the number of individual antigen/antibody reactions occurring in a system. The micro-precipitation test (capillary tube method) gave results of similar accuracy to the LA test (Grossklaus & Walther, 1970). A delipidised aqueous extract of T. saginata

proglottids was used as antigen. However, in slaughter cattle the GP test with a similar antigen only detected 41 per cent of infected animals (Frick & Süsse, 1970). When used with sera from slaughter animals in Chad, the GP test compared unfavourably with other serological techniques (Martin, 1972) and cross reactions occurred with F. gigantica. An aqueous extract of T. saginata cysticerci was used as antigen. With cattle given an experimental oral infection of 100,000 T. saginata eggs at 3 months of age the micro-GP test (Crowle, 1958) first gave a positive reaction 2 weeks pi (Gallie & Sewell, 1974b) This was shorter than the 4-6 weeks taken for the IDH test to give a positive reaction. A borate buffered saline extract of mixed gravid and non-gravid T. saginata strobilae was used as antigen. The antibody levels in these older cattle remained strong for up to 7½ months pi, whereas younger cattle, which had first been infected at a few days old, did not develop strong gel-precipitating antibodies (Gallie & Sewell 1974a).

1.2.6.6. The indirect immuno-fluorescent (IIF) test.

This test has met with mixed results. In one case, (Grossklaus & Walther, 1971) it was not possible to distinguish between infected and non-infected control cattle. A soluble extract of T. saginata strobilae and cyst material fixed on to cellulose acetate paper was used as the antigen matrix. Calamel & Soulé (1972) found that fixed sections of hatched and activated T. saginata embryos gave best results in the IIF test.

Cross reactions occurred with other helminths such as Taenia pisiformis cysticerci, T. hydatigena cysticerci and E. granulosus, but not with the trematodes F. hepatica or D. dendriticum. In an experimental infection the IIF test first gave a positive response between 2 and 4 weeks pi, (Soulé, Calamel, Chevrier & Pontaleon, 1971), the titres were low, reaching a maximum of only 1:160 in cattle given 100,000 T. saginata eggs, and began to tail off by 10-16 weeks pi. Sections of activated T. saginata embryos were used as antigen. The serological response of cattle given a repeat oral infection with T. saginata eggs when antibodies could no longer be detected to the primary infection was similar, but again with low titres (Soulé, Chevrier & Calamel, 1972). However, Machnicka (1973), using hatched and activated T. saginata embryos as antigen, reported positive titres in the IIF test which reached 1:640 in one case and 1:2,560 in another with sera from experimentally infected calves and these titres were usually higher than titres for the IDH test.

Cross reactions occurred with D. dendriticum when the IIF test was used in the ante-mortem diagnosis of T. saginata infections in slaughter cattle (Éuzeby & Dubra, 1970). Sections of T. saginata cysticerci and sections of egg - bearing T. saginata segments were used as antigen.

The main disadvantage of the IIF test is the subjective manner in which the results are read. In a technique which uses fixed sections of helminths as

antigen there is little possibility of using methods of antigen purification to improve results. If on the other hand soluble antigens are fixed onto a matrix (Grossklaus & Walther, 1971) there is the possibility of using antigen purification techniques.

1.2.6.7. Efficiency of ante-mortem diagnosis.

Most of the serological techniques were effective when used to monitor the response of cattle to experimental infection with T. saginata. Possibly the most sensitive is the CF test, followed by the IDH test and the LA test, although Machnicka (1973) reported that the IIF test was slightly more sensitive than the IDH test. The LA and GP tests detected experimental infection earliest at 10 days and 14 days pi respectively. The serological response can remain strong for up to a year pi depending on the age of the calf at infection and the technique used to monitor infection. In virtually every case, where these serological techniques were used to monitor the sera from slaughter cattle, problems were reported due to cross reactions from other helminth infections which caused false positive results. False negative results were encountered with animals with a low parasite burden.

This highlights the main drawbacks in the use of serological techniques in the detection of T. saginata infection in cattle. In cattle which are infected with other helminths and may or may not be infected with T. saginata cysticerci, there is the problem of cross

reactions. Cattle which are immunologically unresponsive to T. saginata infection or cattle which have very low antibody titres, often because they contain few cysts, will give rise to false negative results.

1.2.7. Antigen preparation and purification.

The most common type of antigen used in these serological studies was a delipidised extract of T. saginata, either strobilae or cyst material. This method is possibly acceptable when using the antigen to monitor the response of cattle of known history to experimental infection, but it is not very effective if used to diagnose field infections, as these cattle may be infected with other helminths etc. causing cross reactions. The effectiveness of any particular serological technique in detecting antibodies to T. saginata cannot, therefore, be fully assessed unless the method of antigen preparation is also taken into account. There is a need to develop a purification technique for T. saginata extracts used as antigens in order to obtain less heterogeneous and more defined antigen preparations which would possibly result in greater specificity in the serological tests.

There is not an extensive literature on the fractionation and purification of saline extracts of T. saginata, possibly because of the difficulty in obtaining fresh material of good condition in sufficient quantity. Therefore, a brief review will be given of the techniques and procedures generally in use for the analysis and purification of other helminths, including nematodes,

trematodes and cestodes before dealing with papers relating to T. saginata.

1.2.7.1. Other helminths.

In serological work involving helminths a simple saline extract of the helminth is often used as 'antigen' but in order to obtain a less heterogenous preparation the techniques of acid soluble extraction (Melchner, 1943) and ether extraction (Chaffee et al, 1954) may be used. A general approach to the fractionation and purification of helminth antigens (N.H. Kent, 1963) involved defatting, gel filtration and ion exchange chromatography. These techniques have been applied to the purification and analysis of many helminth antigens and the various techniques used in the preparation and analysis of soluble helminth extracts for use as antigens in immuno-diagnostic tests for nematode, trematode and cestode parasites have been reviewed (J.F. Kent, 1963). The various helminth extracts used as antigens in the study of hydatidosis and the methods used in their characterisation such as electrophoresis, chromatography and gel diffusion have been listed (Kagan & Agosin, 1968).

Immunoelectrophoresis was used as the main analytical tool in an antigenic analysis of extracts of the nematode Trichinella spiralis (Tanner & Gregory, 1961) but neither acid nor alkali extraction nor the isolation of metabolic products yielded antigens different from those found in a buffered saline extract of the adult parasite. An antigen

active in the intradermal skin test was isolated from a homogenate of Dirofilaria immitis by a combination of precipitation with trichloroacetic acid, ion exchange and gel filtration chromatography. The final antigenic extract, was considered to be a protein containing a small amount of carbohydrate (Sawada, Takei, Katamine & Yosimura, 1965).

One of the substances most active in the CF test for Chlonorchis sinensis was a poly-glucose extracted from a saline extract of that helminth by a complex purification procedure involving both ion exchange and gel filtration chromatography (Sawada, Takei, Williams & Moose, 1965).

The different fractions most active in the IDH and CF tests were isolated by fractionation of saline extracts of Schistosoma japonicum using a combination of chromatographic procedures and treatment with pronase (Sawada, Takei, Sato & Sato, 1972). A different procedure was followed to isolate the antigens of less than 200,000 in molecular weight, which were most active in the intradermal skin test.

After analysis by a combination of gel filtration, ion exchange chromatography, immunodiffusion and immunoelectrophoresis, the antigenically active molecules in various extracts from Taenia solium cysticerci were found to have molecular weights greater than 200,000 (Morris, Proctor & Elson - Dew, 1968).

The first fraction from the Sephadex-G200 chromatographed extract of E. granulosus scolices, consisting of large molecules, was satisfactory when used in the soluble antigen fluorescent antibody (SAFA) test, (Gore, Sadun

& Hoff, 1970). Due to cross reactions this antigen could also be used to detect Echinococcus multilocularis infection. The corresponding fraction from hydatid fluid was unsatisfactory, since a large percentage of sera from individuals with parasitic, bacterial and mycotic infections reacted in the test.

If antigens are purified by gel filtration or ion exchange chromatography, it is really just a matter of luck whether the immunologically active molecules are separated from non specific and/or cross reacting components. After fractionation of the extract, there is the time consuming problem of assessing all the different fractions. Immunoadsorption techniques are beginning to be used in the purification of helminth antigens. These techniques should in theory provide a way of preparing specific antigens since they rely on the specificity of the antigen/antibody reaction.

The main problem in purifying sheep hydatid fluid is removing the sheep serum components present in the fluid. Immunoadsorption techniques were used in the purification of a lipo-protein from E. granulosus cyst fluid from sheep (Oriol, Williams, Esandi & Oriol, 1971). A relatively pure E. granulosus antigen was produced using polymerised sera from rabbits tolerant to sheep serum and immunised with crude sheep hydatid fluid (Pozzuoli, Musiani, Arru, Patrono & Piantelli, 1974). On analysis by Sephadex G200 chromatography, the molecular weights of the antigenic components were estimated to be around 400,000

and 150,000. However, there was no net gain in sensitivity although there was increased specificity by the use of purified antigens rather than crude sheep hydatid fluid.

Similarly the use of CNBr activated Sepharose 4B coupled to an IgG fraction of rabbit serum immunised against hydatid fluid fraction "F5" resulted in the purification of "F5" antigen from hydatid fluid. This antigen was considered to be a lipo protein (Bout, Fruit & Capron, 1974). Again there were two antigenic components in the hydatid fluid separable by Sephadex G200 chromatography, one of high molecular weight in the first fraction and the other of a size similar to albumin.

More recently a simplified procedure has been developed to purify hydatid fluid using affinity chromatography on concanavalin A - Sepharose (Con A - Sepharose). Two antigenic components "F4" and "F5" were isolated (Pozzuoli, Piantelli, Perucci, Arru & Musiani, 1975). Con A - Sepharose has a specificity for a variety of polysaccarides and glycoproteins.

Immunoadsorption conducted on CNBr activated Sepharose 4B columns was partially successful in removing cross reacting antigens from whole worm extracts of Anisakis (Filocapsularia Des Longchamps, 1824) larvae. The procedure was most successful when applied to a partially purified extract of perienteric (body) fluid extracted from Anisakis larvae (Sato, Suzuki, Shiraki, Yamashita & Otsuru, 1974). Immunoadsorption techniques have also

been applied to the purification of an extract of Angiostrongylus cantonensis for use in skin tests for that parasite but it has not yet been determined whether this particular antigen would be of value for practical application, (Suzuki, Sato, Yamashita, Sekikawa & Otsuru, 1975).

Immunoadsorption techniques using polymerised sera or sera bound to CNBr activated Sepharose 4B columns is an aspect of helminth antigen purification which has still to be fully exploited but it would appear to have potential in removing cross reacting antigens and other non specific material present in saline extracts of whole parasites.

1.2.7.2. Purification of saline extracts of T. saginata.

In a detailed study on the preparation and chemical analysis of T. saginata, 3 lipid, 3 polysaccaride and 5 protein and nucleoprotein fractions were isolated by chemical fractionation of T. saginata strobilae (Machnicka, 1965). The fractions were compared for antigenic activity in serological tests for patients infected with T. saginata (Machnicka, 1966). A purified polysaccaride fraction had the highest activity in the IDH test and a nucleoprotein fraction in the CF test. Two of the carbohydrate and 3 of the protein fractions contained a single gel precipitin antigen and a carbohydrate fraction isolated at alkaline pH showed 3 delicate gel precipitin lines on testing against sera from experimentally infected calves (Machnicka, 1974). In the tanned cell IDH test a

protein fraction showed highest activity but when untanned cells were used a carbohydrate fraction isolated at alkaline pH was most active. Extracts from T. saginata cysticerci were found to have antigenic components in common with the adult parasite.

Fractions of lyophilised whole T. saginata cysticerci extracted at pH 5.85 and pH 7.2 and protein fractions obtained with 50, 60 and 80 per cent saturated ethanol and 65 per cent saturated ammonium sulphate gave the most satisfactory antigens for use in the LA and IDH tests (Sokolovskaya, 1966).

Cross reactions were encountered in a comparative analysis of saline extracts from T. saginata strobilae, T. saginata cysticerci and extracts of various other helminths (Enyenihi, 1970, pers. comm.). After purification by gel filtration on Sephadex G200 and heat treatment, some of the antigens responsible for the cross reactions with T. saginata were removed or inactivated. The cross reacting components of various helminths have been demonstrated by comparative immunoelectrophoresis studies (Capron, Biguet, Vernes & Alfchain, 1968) using anti-sera raised in immunised rabbits. The number of common components is usually related to the closeness of the taxonomic relationship of any two helminths.

A saline extract of T. saginata proglottids was separated into 8 serologically distinguishable fractions by both gel filtration and ion exchange chromatography (Grossklaus & Walther, 1970). The first fraction from

Sephadex - G75 chromatography of a saline extract of T. saginata contained 3 antigens considered to be of diagnostic importance (Ballad, 1973). More specific results were obtained in the IDH test when the first fraction, obtained from Sephadex - G75 or Sephadex G200 chromatography, of a homogenate of fresh T. saginata cysticerci was used as antigen, rather than a delipidised extract of homogenised T. saginata cysticerci (Bol'shakova, Krasovskaya, Ballad, Moskvin & Sitov, 1975).

Generally, therefore, the methods used in antigen purification can be divided into chemical means of purification such as ether extraction, acid or alkaline extraction, precipitation of carbohydrates with ethanol etc. and the more gentle physico-chemical techniques such as gel filtration, ion exchange chromatography and recently immunoadsorption techniques.

Although many of the procedures used in the isolation of substances from helminths follow the same basic principles no two extraction procedures were identical. This emphasises the individuality of the isolated antigens and the necessity for adapting existing procedures to each individual helminth antigen.

1.2.8. Other parameters.

Several authors have studied the serum protein levels in cattle infected with T. saginata. The serum globulin levels in cattle increased by 25 days pi (Mosina, 1965) and both hypoalbuminaemia and hyperglobulinaemia have been

reported in infected animals (Evranova & Mosina, 1965 and Alferova, 1968). The immunoglobulin levels in the blood of infected animals were determined by the LA test (Leikina & Poletaeva, 1973). The IgM levels rose to a peak titre of 1:16 at 3 months pi, fell at 8 and 13 months and rose again at 14 months. The IgG titre rose to 1:8 by 1.4 months (6 weeks) pi, fell to 1:4 at 3 months pi and rose to 1:32 at 5 months pi. The total protein and total globulin concentration rose significantly and the albumin concentration showed a significant decrease in T. saginata infected calves in the period from 1-11 weeks pi (Gallie & Sewell, 1974b).

Levels of serum alkaline phosphatase, serum transaminases and serum lactate dehydrogenase (E.C. Numbers not specified) remained within normal limits during infection (Dewhirst & Cramer, 1965). Glycogen levels in the liver and skeletal muscles decreased and changes in the metabolism of nucleic acids were noted in cattle during T. saginata infection (Evranova & Mosina, 1965). Cattle with heavy infections of T. saginata showed a depressed general condition accompanied by a lack of appetite and an elevated temperature on the tenth day pi, which lasted several days (Branatov, Stoimenov & Manov, 1974).

Although it is interesting to monitor these general parameters associated with T. saginata infection it is not possible to consider these as a specific response to T. saginata infection because similar responses occur in many other conditions.

CHAPTER 2

2. Materials and Methods.

2.1. The parasite.

The T. saginata used in these experiments were supplied to the C.T.V.M. by Dr. I. Mann of the University of East Africa. Mature and immature T. saginata proglottids were obtained by Dr. Mann from sewage treatment plants in Kenya. On arrival they were washed several times in 0.85 per cent saline solution prior to storage at -20°C until used.

T. saginata eggs were extracted from gravid T. saginata proglottids obtained from infected patients attending dispensaries in Kenya. The eggs were washed several times in 0.85 per cent saline solution containing 100 units of penicillin (Crystapen; Glaxo Laboratories Ltd., Greenford) 100 µg of streptomycin (streptomycin sulphate; Glaxo Laboratories Ltd.) and 2.5 µg of Amphotericin B (Fungizone; E.R. Squibb & Sons Inc. New York, U.S.A.) per ml and stored at 4°C in this saline solution until used.

T. saginata cysticerci were obtained when cattle experimentally infected with these eggs were slaughtered.

2.2. Animals.

2.2.1. Calves.

Four Ayrshire bullocks were purchased from Easter Bush Farm, Midlothian. They were all ear tagged for identification purposes and coded for reference. Calf E1 was 4½ months, E2 was 5½ months, E3 was 11 months and E4

just under 2 months old on arrival. A Friesian heifer (E5), purchased from Mr. P. Fullarton of the Nutrition Department, C.T.V.M., was also just under 2 months old on purchase. These calves were used in two experiments. On arrival they were dosed with the anthelmintic Thiabendazole (Merk, Sharp & Dohme Ltd., Hoddesdon).

2.2.2. Rabbits.

Two black Porton rabbits and one New Zealand white half lop-eared rabbit were purchased from the Centre for Laboratory Animals, Bush Estate, Milton Bridge, Penicuik. The rabbits were 6 months old on arrival and were used to raise rabbit anti-bovine serum globulin.

2.2.3. Goats.

Two adult British Alpine goats were obtained from Dr. S. Al-Samarrae of the Helminthology Department, C.T.V.M. The goats were used to raise goat anti-rabbit serum globulin.

2.2.4. Sheep.

Normal control sheep belonging to Dr. G.R. Scott of the Virology Department, C.T.V.M. were used as a source for whole sheep blood. Both Cheviots and Blackfaced sheep were used.

2.3. Experimental procedures applied to animals.

2.3.1. Oral infection of cattle with T. saginata eggs.

Three Ayrshire castrated bullocks were orally infected

with mature and viable T. saginata eggs (Table 1). The T. saginata eggs used to infect the cattle were supplied by Dr. G.J. Gallie of the Helminthology Department, C.T.V.M. already counted and checked for maturity and viability (2.6.1.1.). The washed and counted eggs were administered to the cattle by means of a 10 ml syringe (Plastipak; Becton, Dickinson & Co. Ltd., Ireland). The eggs in 0.85 per cent saline were sucked up into the syringe and squirted well down the animals throat. The beaker containing the eggs was washed out several times and the washings given to the animal to ensure that all the eggs were adminstered.

Table 1 The numbers of T. saginata eggs used to orally dose three bullocks.

Bullock	Age when infected	Number of T. saginata eggs administered
E1	5 months	60,000
reinfection (E1)	10 months	80,000
E2	6 months	100,000
E3	12 months	100,000

Calves E2 and E3 were also given an intramuscular injection of a saline extract of T. saginata proglottids, in the form of a double emulsion with complete Freund's adjuvant (2.6.1.8.2.), 8 months after the oral infection. A 10 ml

blood sample was taken weekly from each of these calves in order that the serological response to infection could be monitored.

2.3.2. Intramuscular injection of calves with hatched and activated T. saginata eggs.

Two calves (E4 and E5), aged 2 months, were injected intramuscularly (i/m) with approximately 5,000 hatched and activated T. saginata eggs at 4 sites, one on each rump and one on each shoulder.

The eggs were washed, counted and checked for maturity and viability as outlined (2.6.1.1.). The procedure used for hatching and activating the eggs was according to Gallie & Sewell (1970). The technique was slightly modified by the addition of 0.002 M Cysteine hydrochloride to the activation solution (Gallie, pers. comm.). The eggs were injected in 1 ml of 0.85 per cent saline solution and left to develop for 3 months prior to slaughter. During this time 10 ml blood samples were taken each week. On slaughter the injection sites were removed and the cysts retrieved.

2.3.3. Production of antisera.

The method of raising the rabbit anti-bovine serum globulin (Schedule 1) was a slightly modified version of that described by Penhale & Christie (1969) and the method for raising the goat anti-rabbit serum globulin (Schedule 2)

was adapted from one suggested by Herbert (1973). Prior to injection the rabbit and bovine sera were precipitated with 33 per cent ammonium sulphate (2.6.1.6.) to obtain the globulin fraction in a fairly pure form. The protein content was checked by the method of Warburg & Christian (1941).

Schedule 1 - rabbit anti-bovine serum globulin.

Three rabbits were used to produce rabbit anti-bovine serum globulin.

Day 0	5 mg of bovine globulin in incomplete Freund's adjuvant injected i/m into each thigh.
Day 10	As for day 0.
Day 20	1 mg of bovine globulin intravenously (i/v).
Day 23	2 mg of bovine globulin i/v.
Day 27	4 mg of bovine globulin i/v.
Day 32	5 ml blood sample taken and the gel precipitin titre checked.
Day 40	40 ml blood sample taken.

The rabbits were again stimulated after a rest period of three months by a repeated programme of i/v injections.

Schedule 2 - goat anti-rabbit serum globulin.

Two goats were used to produce goat anti-rabbit serum globulin.

Day 0	25 mg of rabbit globulin in Freund's incomplete adjuvant were injected i/m into each rump.
Day 21	As for day 0.

Day 28 10 ml blood sample taken and the gel precipitin titre checked.

Day 34 500 ml blood sample taken.

2.4. Collection of blood samples and extraction and storage of serum samples.

2.4.1. Cattle and goats.

Blood samples were taken from the jugular vein of cattle and goats using 10 ml vacutainers (Becton, Dickinson & Co. Ltd., Grenoble, France) and 1½" vacutainer needles. Large blood samples of approximately 500 ml were also taken from the jugular vein using a blood transfusion needle with a siliconised tube attached. A large wide necked jar was used to collect the blood sample.

2.4.2. Rabbits.

With rabbits the blood samples were taken from the lateral ear vein. The right ear was generally used for injection purposes and the left for taking blood samples. A 1" 20 gauge hypodermic needle (Yale Microlance; Becton, Dickinson & Co. Ltd.) was inserted into the dilated vein and the blood allowed to drip through the needle and into a universal bottle. Both small test samples of about 5 ml and larger samples of up to 30 ml were taken by this method.

2.4.3. Sheep.

Blood samples were taken from sheep using 1" 20 gauge hypodermic needles (Yale Microlance; Becton, Dickinson & Co. Ltd.) and 10 ml plastipak syringes (Becton, Dickinson &

Co. Ltd.). The blood was taken from the jugular vein and quickly transferred to a sterile universal bottle containing 10 ml of sterile Alsever's solution (Kent, Bukantz & Rein, 1966). The resulting suspension was allowed to stabilise at 4°C for a week before use.

2.4.4. Extraction and storage of serum samples.

After a blood sample had been taken, the blood was left at 37°C for 30 minutes to permit clot formation and then left overnight at 4°C to ensure that the clots were retracted as fully as possible. The following day the clots were removed and the serum centrifuged for 30 minutes at 4°C and 2,500 g. The clear serum was transferred to plastic vials (Luckam Ltd. Labro Works, Burgess Hill, Sussex) and stored at -20°C.

With large samples of up to 500 ml, once the clot had formed it was gently cut into four pieces with a sharp knife. This enabled the large clot to retract more efficiently. Serum from large samples was usually centrifuged for two 30 minute periods instead of 1 to ensure complete removal of the red blood cells.

2.5. Information on and the sources of additional serum supplies.

Dr. G.J. Gallie supplied sera from some of his experimentally infected cattle. Weekly serum samples were obtained from 3 cattle (E6, E7 and E8) orally infected with T. saginata eggs at 3 months of age and also

2 control calves (N/1 and N/2). Both groups were challenged with 150,000 eggs at 13 months old. The details of this experiment were given by Gallie & Sewell (1974b).

Sera was also supplied from an experiment (Gallie & Sewell, 1974a) in which there were 15 cattle divided into 3 groups. The first group (E9 - E13) were serially infected calves, who were orally dosed with 10,000 T. saginata eggs at 2-3 days of age and thereafter with 500 eggs weekly. A single infection group (E14 - E18) were orally dosed with 10,000 T. saginata eggs at 2-3 days of age and the control group (N3 - N7) were given no eggs at all until all the cattle were challenged orally with 50,000 eggs at 1 year old.

Large volumes of sera were available as positive controls from 3 cattle infected orally with 30,000 T. saginata eggs at 9 months of age. The samples were taken 3 months after infection and were labelled E19, E20 and E21 for reference. Sera was also available from 3 calves given a series of i/m injections with a saline extract of homogenised T. saginata proglottids in Freund's complete and incomplete adjuvant when they were 6 months old (Gallie & Sewell, 1976). The serum samples were taken when the cattle were 1 year old and were labelled E22, E23 and E24 for reference.

A pooled serum sample was obtained from 3 cattle infected orally at 6 months of age, one with 250,000 eggs and the others with 500,000 eggs. The serum sample

was taken 4 months after oral infection (Gallie unpublished). This serum was labelled E25 for reference.

Normal bovine serum was obtained in bulk from several experimental control cattle of known history and free from T. saginata infection, for use as control sera in serological tests. Large samples of sera from apparently healthy farm and slaughter cattle were used in the preparation of bovine globulins for use as immunoadsorbents.

A large pooled serum sample was taken from 2 experimental control calves of Dr. G.J. Gallie. These calves (N8 and N9) were both 6 months old at the time of sampling. Serum was taken from a calf belonging to Mr. A. Trees of the Protozoology Department, C.T.V.M. This calf (N10) had been splenectomised at the age of 3 months and the blood sample was taken when it was 1 year old.

Normal serum was obtained from an 8 year old Ayrshire cow (N11) belonging to Mr. P. Fullarton, which had been culled from a dairy herd because it was infertile. Large samples of normal serum were taken from two Ayrshire cows (N12 and N13) about 5 years old from Easter Bush Farm, Midlothian. This farm had no history of T. saginata infection. In addition serum was obtained from apparently normal cattle (N14 and N15) slaughtered at Gorgie Abbatoir, Gorgie Road, Edinburgh.

In cooperation with Mr. S.M. Richmond of the Department of Agriculture and Fisheries for Scotland, samples were taken from 24 cattle (A1-A24) on a farm in Fife which had a recent outbreak of T. saginata infection.

Mr. Richmond also supplied the limited information available on these cattle, which were over 1 year old when sampled. Three of the calves sampled (A11, A17 and A18) were found on slaughter and inspection by routine meat inspection techniques to be lightly infected with T. saginata. The calves had been kept on the farm for at least a year to 18 months past the normal time for slaughter before being sold. This was to allow any cysts present to degenerate.

As a control samples were taken from 14 cattle (B1-B14) on another farm in Fife which had no history of T. saginata infection and from 11 (C1 - C11) year old Ayrshire heifers from Easter Bush Farm, Midlothian. There was no slaughter information available for these control farms but these had no history of T. saginata infection.

Serum samples have also been obtained from cattle abroad including both experimentally infected cattle and cattle naturally infected with T. saginata when on a farm in Holland. Imported sera had to comply with Scottish Animal Health Department regulations and were consequently treated with 0.2 per cent. Betapropriolactone and then heated at 56°C for 30 minutes prior to dispatch.

Dr. F.J. Ruitenberg of the Rijks Institut voor de Volksgezondheid, Bilthoven, Holland supplied serum samples from five 2 year old cattle (D1 - D5). There were four females and 1 male. These cattle were from a farm which had an outbreak of T. saginata cysticercosis. On slaughter D1 and D5 were not found to be infected with T. saginata but D2 had many calcified cysts and D3 and D4 were lightly

infected. These cattle were slaughtered approximately 9 months after the serum samples were supplied.

Dr. M. Walther of the Institut für Veterinarmedizin, Robert von Ostertag Institut, Berlin sent serum samples from 4 experimental calves which were infected orally with T. saginata eggs at 2 - 3 months of age. Two of these calves (E26 and E27) were given 100,000 eggs, one 500,000 (E28) and the last (E29) was given 1,000,000 eggs. The serum samples were from varying periods in the infection ranging from 1 day prior to infection up to 60 days pi. He also included sera from control cattle. One sample (N16) was from a 3 year old, apparently healthy slaughter house beast and the other (N17) from a 6 month old uninfected control calf.

Dr. T. Kassai of the Veterinary University, Helminthological Research Laboratory of the Department of Parasitology, Budapest, Hungary supplied serum from 2 cattle (E30 and E31), given an experimental oral infection with 100,000 T. saginata eggs. The serum samples were taken from week 7 to week 13 pi.

Mr. P.D. Le Riche of the Helminthology Department, C.T.V.M. supplied serum from a Black faced sheep which was given a per os infection of 1,600 viable T. ovis eggs when a year old. The serum sample was taken 3 months later when the sheep was slaughtered. There was a 9 per cent. take of T. ovis cysticerci, 144 dead cysts were found in the carcase.

2.6. Preparative procedures.

2.6.1. General techniques.

2.6.1.1. Treatment of T. saginata eggs.

T. saginata eggs were counted using Eel worm counting chambers and the eggs were checked for maturity by microscopic examination. Mature eggs were distinguishable by their thick brown embryophore. Only samples of eggs containing upwards of 90 per cent mature eggs were used. The eggs were also checked for viability by microscopic examination but first the embryophore was digested off with pepsin solution (Gallie & Sewell, 1970). Any vacuolated or obviously degenerate eggs were considered to be non-viable. Only samples containing 80 - 90 per cent viable eggs were used for infecting cattle.

2.6.1.2. Preparation of a saline extract of T. saginata proglottids (SE).

A sample of mixed mature and immature proglottids was blotted with filter paper to remove any excess fluid and cut up finely with scissors. Four ml of 10 per cent borate buffered saline pH8.45 (BBS) was added to each gram of chopped T. saginata proglottids. The stock borate buffer was made by dissolving 6.184g of boric acid, 9.536g of sodium tetra borate and 4.384g of sodium chloride in distilled water and making the volume up to 1 litre. The pH was between 8.4 and 8.5. The 10 per cent BBS solution was made by adding 100 ml of BBS stock to 900 ml of 0.85 per cent saline solution.

The material was kept on an ice bath while being homogenised for 4 x 1 minute periods on a laboratory homogeniser (Silverson Machines Ltd., Waterside, Chesham, Bucks.) followed by 2 x 1 minute periods on an MSE 100 watt Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London) at an amplitude of 8 μm. The homogenate was made up to 10 ml with 10 per cent BBS and 100 units of penicillin (Crystapen; Glaxo Laboratories Ltd.) and 100 μg/ml of streptomycin (streptomycin sulphate; Glaxo Laboratories Ltd.) were added to prevent bacterial growth before the solution was left to extract overnight at 4°C. The suspension was centrifuged for 30 minutes at 4°C and 2,500g and the supernatant (SN1) retained and stored separately at -20°C until use. The precipitate was resuspended in 4 ml of 10 per cent BBS and the procedure of homogenising, sonication etc. repeated three additional times to produce SN2, SN3 and SN4 which were all stored separately at -20°C until used.

These four extractions were made in order to maximise the amount of antigen obtained from the material available, as this was limited.

All four supernatants were pooled and centrifuged for 30 minutes at 2,500g and 4°C to remove any remaining particulate material. The SE was then concentrated on Millipore 142 mm Laboratory Ultrafiltration Equipment using a Pelicon Ultrafiltration Membrane, Type PSAC with a nominal molecular weight cut off at 1,000 (Millipore (UK) Ltd., Wembley, Middlesex). The final concentration gave approximately

1 ml of SE from 1 g of proglottids.

The concentrated SE was ultracentrifuged at 100,000 g for 30 minutes at 4°C on a Beckman L2-65 B Ultracentrifuge (Beckman Instruments Ltd., Glenrothes, Fife.). After estimating the protein content of the supernatant by the Folin technique (2.7.1.) and the carbohydrate content by the anthrone reaction (2.7.4.) the SE was stored in vials at -20°C. The SE used in the preliminary studies of the antigen was only extracted once in 10 per cent BBS and not four times, and it was neither concentrated nor ultracentrifuged.

2.6.1.3. Extraction of T. saginata cyst fluid.

T. saginata cysticerci were removed from the carcases of experimentally infected cattle at post-mortem examination. They were dissected out carefully from the surrounding capsule and then washed several times in 0.85 per cent saline solution. Any broken cysts were placed in a separate container. After washing the whole cysts the maximum possible amount of saline was sucked off by pipette. The cyst walls were cut open and the cyst fluid was allowed to drain out. This fluid was collected using a pipette. Following centrifugation at 2,500 g and 4°C for 30 minutes the cyst fluid was stored in plastic vials at -20°C until used. An estimate was made of the protein and carbohydrate content.

2.6.1.4. Preparation of a saline extract of T. saginata cysticerci scolices and membranes.

The scolices and cyst membranes were washed several times in 0.85 per cent saline to remove any traces of cyst fluid. A saline extract was then made from the scolices and membranes by the same method as that outlined for the preparation of SE (2.6.1.2.). As the scolices were difficult to homogenise, the preparation was ground up by mortar and pestal rather than being homogenised. After extraction the saline extract was stored in plastic vials at -20°C until use. An estimate was made of the protein and carbohydrate content.

2.6.1.5. Preliminary work on SE.

2.6.1.5.1. Heat treatment.

Aliquots of SE were subjected to heat treatment by incubation in waterbaths at 50°C, 65°C, 75°C and 100°C for 30 minutes. After this treatment the preparations were centrifuged for 15 minutes at 1,500g and 4°C. The supernatants were kept for analysis. A slight precipitate was formed, which increased with the incubation temperature, but this precipitate was discarded.

2.6.1.5.2. Precipitation with trichloroacetic acid.

An aliquot of SE was precipitated with trichloroacetic acid (TCA) as described by Dawson, Elliot, Elliot & Jones (1969). The TCA was added dropwise to a final

concentration of 0.25 M, which is sufficient to ensure almost complete precipitation of proteins. The solution was stirred for 30 minutes on a Luckam rotator (Luckam Ltd.) and then centrifuged for 15 minutes at 1,500 g and 4°C. The supernatant was dialysed overnight in a dialysis bag of visking tubing (Scientific Instruments Centre, London) against running tap water. The precipitate was also dialysed after it had been taken into solution in the original sample volume in phosphate buffered saline (PBS) pH. 7.3 (Oxoid phosphate buffered saline tablets; Oxoid Ltd., London).

2.6.1.5.3. Precipitation with ammonium sulphate.

Aliquots of SE were placed in dialysis bags of visking tubing and the sealed bags placed into solutions of ammonium sulphate calculated to give a final concentration in the bags of 20, 40, 60, 80 and 100 per cent saturated ammonium sulphate. The solutions were mixed on a Luckam rotator overnight at room temperature. The contents of the bags were then centrifuged for 15 minutes at 1,500 g at room temperature. The precipitates were each redissolved in the original sample volume in PBS pH 7.3 and along with the supernatants were dialysed against PBS pH 7.3 at 4°C until all the sulphate ions were removed (2.6.1.6.).

2.6.1.6. Ammonium sulphate precipitation of serum gamma globulins.

Samples of serum were precipitated with ammonium sulphate using a slightly modified version of the method outlined by Campbell, Garvey, Cremer and Sussdorf (1964). Procedure:- With constant stirring 25 ml of a saturated solution of ammonium sulphate (760 g ammonium sulphate / litre of water at room temperature) were added dropwise to 50 ml of serum. During the first stages all the precipitate from one addition was allowed to dissolve before adding further drops. After all the ammonium sulphate had been added the pH of the suspension was checked and adjusted to 7.8 with NaOH.

Small samples were adjusted with 0.5 - 1.0 M NaOH and large samples of 100 ml or more, with 4.0 - 5.0 M NaOH. If strong alkali is added to small samples it may result in excess of alkali with subsequent denaturation of the protein, while the use of weak alkali on large samples may dilute the system to the point where the gamma globulins will begin to dissolve.

This procedure resulted in a 33 per cent saturated solution of ammonium sulphate but any desired concentration could be achieved by adjusting the relative volumes of protein solution and saturated ammonium sulphate.

The suspension was stirred for at least 2 hours at room temperature or overnight at $4^{o}C$, to avoid mechanical trapping of serum components, other than gamma globulins, in

the precipitate. Following centrifugation for 30 minutes at 1,500 g, the supernatant was discarded and the precipitate dissolved in enough 0.85 per cent saline solution to restore the volume of the original serum sample.

Two additional precipitations were made exactly as above, in order to obtain as pure a sample as possible. The precipitate from the third precipitation was dissolved in PBS pH 7.3 and made up to half the original serum volume to concentrate the solution.

Ammonium sulphate was removed by dialysis against several changes of diluent over several days at $4^{o}C$. Generally the solution was dialysed against PBS pH 7.3 but if the serum was to be fractionated by ion-exchange chromatography, it was dialysed against the appropriate starting buffer. The dialysate was changed night and morning and checked for the presence of sulphate ions by adding a drop of HCl and a few drops of 2 per cent $BaCl_2$ to a small aliquot of dialysate. If sulphate ions were present a white precipitate of barium sulphate formed. Complete dialysis was indicated by a negative test for sulphate ions.

2.6.1.7. Concentration of solutions.

2.6.1.7.1. Dialysis against a solution of polyethylene glycol.

Serum-protein or antigen samples were concentrated by dialysis against polyethylene glycol (carbowax) 6,000

(BDH Chemicals Ltd., Poole, Dorset), using visking tubing, a cellulose membrane with an average pore size of 24 nm, which allowed globular molecules of molecular weight below about 20,000 to pass through. The carbowax was generally prepared as a 40 per cent (w/v) solution (Clausen, 1969) in 0.85 per cent NaCl or in an appropriate buffer.

A volume of the sample to be dialysed was transferred to a washed dialysis bag, made by tying a knot in a length of wet Visking tubing and the bag placed in cold carbowax solution kept at 4°C. The sample was usually sufficiently concentrated in about 12 hours. If the volume of sample to be concentrated was very large, or it was necessary to concentrate the sample quickly, an increased concentration of carbowax was used.

2.6.1.7.2. Lyphogel.

Lyphogel (Gelman Instruments Co., Hawksley and Sons Ltd., Sussex), which is a polyacrylamide gel for macromolecular concentration, was used to concentrate small serum samples up to five fold. The procedure followed was that recommended by the manufacturer.

2.6.1.8. Preparation of adjuvants.

2.6.1.8.1. Saline in oil emulsion.

Freund's incomplete adjuvant was prepared after the method described by Herbert (1974). Equal quantities of antigen solution and a 9:1 oil-arlacel A mixture (Difco

Bacto Adjuvant, incomplete Freund, Difco Laboratories, Detroit, Michigan, U.S.A.) were placed in two vials. The emulsion was formed by vigorously injecting the antigen solution into the oil phase a small quantity at a time. Between each addition of antigen solution the emulsion was shaken briskly. Finally it was recycled several times through the syringe and needle in order to increase the dispersion of the antigen phase.

The finished emulsion was tested by allowing a drop to fall into a beaker of cold water. (The initial drop may spread over the surface but subsequent drops should remain discrete.) The emulsion was only used if it was of the correct type.

2.6.1.8.2. Double emulsion.

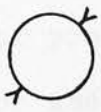
A complete Freund's double emulsion was made according to Herbert (1974) by first preparing a saline-in-oil emulsion as is described above, except that in this case the oil contained mycobacteria (Difco Bacto Adjuvant, complete Freund, Difco Laboratories.). To this primary saline-in-oil emulsion was added twice its volume of saline containing 2 per cent Tween 80 (polyoxyethylene sorbitan mono-oleate; Sigma Chemical Co. Ltd., Kingston-upon Thames, Surrey). The mixture was shaken by hand to disperse the saline-in-oil emulsion into little drops and then forced through a narrow gauge syringe needle several times to reduce the size of the drops still further. Before use the

emulsion was checked under the oil immersion lense of a microscope to ensure that there was a mass of tiny saline droplets inside every oil drop.

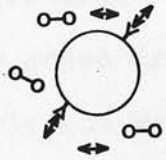
2.6.1.9. Immunoadsorption.

The immunoadsorbent technique is commonly used to isolate antibodies or antigens (Fuchs & Sela, 1974). The technique relys on the specificity of the antigen / antibody reaction. Many immunoadsorbents have been described but two methods commonly used are cross linking antigens or antibodies with glutaraldehyde (Avrameas & Ternynck, 1969) or attaching antigens, haptens or antibodies to Sepharose (Porath, Axen & Ernback, 1967).

Depending on the molecules required, either the antigen or antibodies are first insolubilised by one of the various techniques available (Fuchs & Sela, 1974), thus forming the immunoadsorbent (Figure 1). After a suitable washing procedure the test sample is applied to the immunoadsorbent and the specific components in the sample are adsorbed. Following another wash to remove non adsorbed material, the adsorbed material can be eluted from the immunoadsorbent by changing the buffering conditions. The technique can be carried out either as a batchwise procedure (2.6.1.9.2. and 3.) or in a chromatographic column (2.6.2.3.).

Stage 1.  Prepared immunoadsorbent showing binding sites (≻)

Stage 2. Washing the immunoadsorbent

Stage 3.  Addition of the sample containing specific (⇹) and non-specific (o–o) components

Stage 4.  Washing the immunoadsorbent

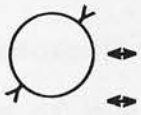
Stage 5.  Elution of adsorbed components by changing the buffering conditions

Figure 1. A schematic representation of the immunoadsorbent technique.

2.6.1.9.1. Preparation of glutaraldehyde insolubilised protein and whole serum.

Solutions of protein and whole serum samples were insolubilised for use as immunoadsorbents following the method of Avrameas & Ternynck (1969). A solution containing 50 mg/ml protein in 0.1 M phosphate buffer, pH 7.0 was stirred gently with a magnetic stirrer. For every 1 ml of protein solution 0.2 ml of a 2.5 per cent aqueous solution of glutaraldehyde, Sigma type IV (Sigma Chemical Co. Ltd.) was added dropwise. A gel formed almost instantaneously and was left to stand at room temperature for 3 hours.

When polymerising whole serum, 10 ml amounts were first dialysed overnight at $4^{o}C$ against 0.85 per cent saline solution. To the dialysed serum, 1 ml of 1.0 M phosphate buffer, pH 7.0 was added and the pH of the solution adjusted to pH 7.0 if necessary. Three ml of a 2.5 per cent aqueous solution of glutaraldehyde, Sigma type IV (Sigma Chemical Co. Ltd.) was added as above. The gel, which was formed after 20 minutes was allowed to stand for 3 hours at room temperature.

The insolubilisation of the protein was shown to be complete by dispersing the material in a few ml of distilled water, centrifuging for 15 minutes at 1,500 g and then checking the supernatant for protein by the method of Warburg & Christian (1941). If the insolubilisation was complete, no protein was found in the supernatant.

Any components in the immunoadsorbent which were soluble in the elution buffer to be used were removed. For this purpose 100 mg of immunoadsorbent was dispersed in 40 ml of 0.2 M phosphate buffer, pH 7.2 and homogenised in small amounts in a glass homogeniser. The resulting suspension was centrifuged for 15 minutes at 1,500 g and 4°C. This washing procedure was repeated three times. Then the insolubilised protein was suspended in 40 ml of eluting buffer, centrifuged and the supernatant discarded. Following one more wash in the eluting buffer, the immunoadsorbent was again washed with 0.2 M phosphate buffer, pH 7.2 until the supernatant showed no absorption at 280 nm; this usually took three further washings.

2.6.1.9.2. Preparation of specific rabbit anti-bovine IgG for enzyme conjugates.

A 33 per cent ammonium sulphate fraction of bovine serum was further purified by DE52 cellulose chromatography using 0.01 M phosphate buffer, pH 6.8 after the method used by Engvall & Perlmann (1972). The globulin fraction which was eluted in the break-through peak was further fractionated on a Sephadex G200 column, to remove any large or small molecular weight contaminants. The globulin fraction was concentrated by dialysis against carbowax and the protein content checked by the method of Warburg & Christian (1941). The purity of the fraction was checked by immunoelectrophoresis against rabbit anti-whole bovine

serum (prepared by Dr. A. Luckins of the Department of Protozoology, C.T.V.M.). Only the gel precipitin lines corresponding to globulins were present. The immunoadsorbent was then prepared by cross linking the purified bovine IgG with glutaraldehyde (2.6.1.9.1.).

The specific rabbit anti-bovine IgG was prepared according to Avrameas & Ternynck (1969). Appropriate volumes (2.7.9.) of serum from rabbits immunised with a 33 per cent ammonium sulphate fraction of normal bovine serum and prepared bovine IgG immunoadsorbent were mixed, stirred gently for 30 minutes at room temperature and centrifuged at 1,500 g for 15 minutes. The precipitate was resuspended in PBS pH 7.3 in 3-4 times the volume of antiserum used and again centrifuged, as above. After 4 washes the supernatant had an absorption of less than 0.04 at 280 nm.

The adsorbed rabbit anti-bovine IgG was eluted from the immunoadsorbent with 0.1 M glycine/HCl buffer, pH 2.8. To do this the immunoadsorbent was suspended in 4 ml of eluting buffer, the suspension was stirred for 5 minutes at room temperature, centrifuged for 15 minutes at 1,500 g at $4^{o}C$ and the supernatant retained. This operation was repeated three times. The pooled supernatants were filtered through a 0.45 µ Millipore filter (Millipore (U.K.) Ltd., Millipore House, London) and dialysed against PBS pH 7.3 for 2 days with several changes of buffer. After dialysis the protein content was estimated by the method of Warburg & Christian (1941) and the solution of

specific rabbit-anti-bovine-IgG was adjusted to contain 5 mg of protein / ml.

The immunoadsorbent was stored in the presence of 0.001 per cent NaN_3 after it had been washed once more with eluting buffer and then several times with 0.85 per cent NaCl.

2.6.1.9.3. Purification of SE by immunoadsorption.

2.6.1.9.3.1. Preliminary experiments.

For these preliminary experiments immunoglobulins were prepared by 33 per cent saturation with ammonium sulphate and then further purified by gel filtration through Sephadex G200. The globulin and macroglobulin fractions were pooled and concentrated by dialysis against carbowax. Immunoadsorbents were prepared by cross linking the immunoglobulins from the sera of two experimental cattle (2.6.1.9.1.).

One calf (E21) had been infected orally with T. saginata eggs and the other (E24) had been given a series of intramuscular injections with homogenised T. saginata proglottids.

Experiment 1a.

The same procedure was used for both immunoadsorbents. A 300 mg batch of prepared immunoadsorbent was split into five 60 mg aliquots which were then prepared as described (2.6.1.9.1.) except that the elution buffers used were 0.1 M glycine / HCl buffer, pH 2.8, 5 M NaI and 2.5 M NaI in 0.05 M Tris (hydroxymethyl) methyl amine / HCl

buffer, pH 9.0 and 5 M $MgCl_2$ and 2.5 M $MgCl_2$ in 0.05 M Tris/HCl buffer pH 7.0. For the aliquots of immunoadsorbent used with the latter two elution buffers unbuffered saline was used throughout the whole operation. The above buffers were suggested by Avrameas & Ternynck (1969).

The aliquots of immunoadsorbent were mixed gently with an appropriate volume (4 ml) of SE for 30 minutes at room temperature and then centrifuged at 1,500 g for 15 minutes. The supernatants were collected and labelled SN 1-10 and according to their elution buffer. The aliquots of immunoadsorbent were washed as described previously. Any adsorbed material was eluted using the five different elution buffers one for each aliquot. Five ml of the appropriate elution buffer was mixed gently with the immunoadsorbent at room temperature for 30 minutes, the supernatant obtained after centrifugation at 1,500 g for 15 minutes was retained. This operation was repeated three times and the appropriate supernatants pooled. Following filtration through a 0.45 µ Millipore filter (Millipore (U.K.) Ltd.) and dialysis against PBS pH 7.3 the pooled supernatants were concentrated back to 2 ml, twice the volume of the original sample. The samples were stored for analysis labelled SN 11 - 20 and according to their elution buffer. Following a further wash in eluting buffer and then several washes in saline the immunoadsorbents were stored at $4^{o}C$ with 0.02 per cent NaN_3 to prevent bacterial contamination.

Experiment 1b.

Two 250 mg samples of the immunoadsorbents prepared from the sera of calves E21 and E24 (2.6.1.9.3.) were prepared for use according to the methods already outlined (2.6.1.9.1.) except that they were each first treated with 0.1 M glycine / HCl buffer, pH 2.8 and then following several washes with PBS pH 7.3 with 5 M NaI in 0.05 M Tris / HCl buffer, pH 9.0.

Appropriate volumes of SE were mixed with the immunoadsorbents as previously described. Any adsorbed material was eluted first with 0.1 M glycine / HCl buffer, pH 2.8. The immunoadsorbents were washed 3 times with PBS pH 7.3 and any further adsorbed material eluted with 5 M NaI in 0.05 M Tris / HCl buffer, pH 9.0. As previously the elution was repeated 3 times for each buffer. The appropriate supernatants were pooled, filtered, dialysed against PBS pH 7.3 and then concentrated back to twice the original sample volume. The fractions were stored at $4^{o}C$ with 5 per cent egg albumin, Sigma type V (Sigma Chemical Co. Ltd.) for stabilisation.

This procedure resulted in three supernatants for each immunoadsorbent:- One supernatant (SN1), eluted with the 0.1 M glycine / HCl buffer, pH 2.8; a supernatant eluted with the 5 M NaI in 0.05 M Tris / HCl buffer, pH 9.0; and the non-adsorbed material.

2.6.1.9.3.2. Experiment 2.

Immunoadsorbents were prepared for this experiment from sera which had previously been precipitated with 40 per cent ammonium sulphate. The procedure used was as described previously (2.6.1.9.1.). Three batches of immunoadsorbent were prepared, 1 from a pool of normal bovine sera (N14 and N15), 1 from serum from a sheep experimentally infected with T. ovis and finally, 1 from a pool of sera from cattle given per os experimental infections with T. saginata eggs. The pool consisted of equal volumes of serum from calves E1, E2, E3 and E25 taken when the serum antibody levels were high. An equal volume of a pool of serum, from calves E6, E7 and E8, made from weekly serum samples taken all the way through the experimental infection, was also included in this pool.

All three immunoadsorbents were prepared for use according to the method described in experiment 1b (2.6.1.9.3.1.), using the two elution buffers, glycine / HCl buffer, pH 2.8 and 5 M NaI in 0.05 M Tris / HCl buffer, pH 9.0.

An appropriate volume of SE was mixed for 30 minutes with each of the immunoadsorbents in succession. Following each mixing the immunoadsorbent was centrifuged at 1,500 g for 15 minutes at room temperature. The aliquot of SE was first mixed with the normal bovine serum immunoadsorbent, followed by the immunoadsorbent prepared from sheep serum. Finally the supernatant was mixed with the immunoadsorbent prepared from the serum from the cattle

orally infected with T. saginata eggs.

Following the routine washing procedure the adsorbed components were eluted from each of the immunoadsorbents, first with the glycine / HCl buffer, pH 2.8 and then with the 5 M NaI in 0.05 M Tris / HCl buffer, pH 9.0. This procedure was carried out as described for experiment 1b (2.6.1.9.3.1.).

The appropriate supernatants were pooled, filtered, dialysed against PBS pH 7.3 and concentrated back to twice the original sample volume. The fractions were stored at -20°C until used but in this case albumin was not used for stabilisation.

This procedure resulted in 7 fractions. Two were eluted from the normal bovine serum immunoadsorbent SN1 at pH 2.8 and SN2 at pH 9.0, 2 were eluted from the sheep serum immunoadsorbent SN3 at pH 2.8 and SN4 at pH 9.0 and 2 were eluted from the T. saginata infected bovine serum immunoadsorbent SN5 at pH 2.8 and SN6 at pH 9.0. The non-adsorbed material was labelled SN7.

2.6.1.10. Conjugation of antibody to alkaline phosphatase.

The enzyme alkaline phosphatase was conjugated to purified rabbit anti-bovine-globulin (2.6.1.9.2.) with glutaraldehyde by the method of Avrameas (1969). The enzyme preparation used was Sigma Type VII (Sigma Chemical Co. Ltd.), of specific activity 520-990 units / mg.

One unit will hydrolose 1.0 µ Mole of p-Nitrophenyl phosphate to p-Nitrophenyl per minute at 37°C and pH 10.4.

The enzyme is supplied in suspension in 3.2 M ammonium sulphate and 0.3 ml of this suspension, containing 1.5 mg of alkaline phosphatase was centrifuged at 4°C for 10 minutes at 1,500 g. The supernatant was discarded and the pellet mixed with 0.1 ml of a solution of specific rabbit anti-bovine-IgG antibodies containing 5 mg of protein / ml. After dialysis overnight against PBS pH 7.3, 10 µl of glutaraldehyde, Sigma type IV (Sigma Chemical Co. Ltd.) were added to a final concentration of 0.2 per cent After 2 hours further dialysis at room temperature, the solution was diluted to 1 ml and again dialysed against PBS pH 7.3 overnight at 4°C.

The conjugate was fractionated on a Sephadex G-200 column to remove any unconjugated globulin and excess glutaraldehyde. The alkaline phosphatase conjugated globulin present in the first peak eluted from the column was pooled and concentrated by dialysis against polyethylene glycol. The protein content of the concentrated conjugate was estimated by the method of Warburg & Christian (1941). The conjugate was made up at 100 µg/ml in a solution containing 5 per cent egg albumin, Sigma type V, (Sigma Chemical Co. Ltd.) for stabilisation in 0.05 M Tris/HCl buffer pH 8.0, with 0.001M $MgCl_2$ and 0.02 per cent NaN_3. Conjugates thus prepared were stored at 4°C.

2.6.1.11. Conjugation of antibody to horse radish peroxidase.

Horse radish peroxidase was conjugated to purified rabbit anti-bovine-globulin by the method of Kawoi & Nakane (1973). The enzyme preparation used was Sigma type VI, (Sigma Chemical Co. Ltd.), RZ approximately 3.0, of specific activity 250 - 330 Purpurogallin (20 second) units/mg. One Purpurogallin (20 second) unit = formation of 1 mg of Purpurogallin in 20 seconds from Pyrogallol at pH 6.0 and 20°C.

Five mg of horse radish peroxidase (HRPO) were dissolved in 1 ml of 0.3 M $NaHCO_3$ and to this were added 0.1 ml of 1 per cent fluorodinitrobenzene in absolute ethanol. The solutions were mixed together at room temperature for 2 hours (Sanger, 1945). This reaction blocks the α- and ε-amino groups of the HRPO. The carbohydrate moiety of the HRPO was oxidised in the dark overnight at 4°C by adding 1 ml of 0.05 M $NaIO_4$ in distilled water (Neuberger & Marshall, 1966). Next morning the oxidation was stopped by the addition of 1 ml of 0.16 M ethanediol in distilled water and the solution was dialysed against several changes of 0.01 M carbonate buffer, pH 9.5. These reactions result in HRPO-aldehyde.

The protein was conjugated to the HRPO-aldehyde at pH 9.5. Five mg of the specific rabbit anti-bovine IgG (2.6.1.9.2.) were dissolved in 1 ml of 0.01 M carbonate buffer, pH 9.5, and to this was added the dialysed HRPO-aldehyde. The solutions were allowed to react at room temperature for 3 hours. This was the same length of time

Avrameas (1969) allowed for the conjugation of enzymes to proteins with glutaraldehyde. The reacted substances were concentrated with polyethylene glycol to a volume of 5 ml and fractionated on a Sephadex G200 column, using 0.1 M Tris / HCl buffer, pH 8.0, containing 1.0 M NaCl, to remove any unconjugated enzyme and protein. The first peak eluted from the column contained the conjugated material. It was pooled, concentrated by dialysis against polyethylene glycol and dialysed against PBS pH 7.3.

The protein content of the concentrated conjugate was estimated by the method of Warburg & Christian (1941) and adjusted to a solution containing 1 mg of protein / ml in PBS pH 7.3 with 5 per cent egg albumin, Sigma type V (Sigma Chemical Co. Ltd.) for stabilisation and 0.02 per cent NaN_3 to prevent bacterial contamination. Only half the total protein used was recovered as conjugated material. This was less than the 90 per cent obtained by Kawoi & Nakane (1973) but fractionation procedures always result in loss of material. The conjugate was stored at $4^{o}C$.

Unfortunately when made in this way and stored at $4^{o}C$, the conjugate lost its activity within 3 months. A later paper (Nakane, 1975) modified the procedure by adding 5 mg of sodium borohydride to the HRPO - aldehyde - globulin complex. The mixture was left at $4^{o}C$ for 4 hours or overnight and then dialysed against PBS pH 7.3 before fractionation by Sephadex G200 gel filtration. The sodium borohydride stabilised the Schiffs' base which binds the peroxidase molecules to the immunoglobulin. This procedure has a satisfactory effect on the stability of the enzyme conjugate (Lehner, 1977). An

alternative /

which latterly became available was to purchase HRPO conjugated antisera from Nordic Immunochemical Laboratories, Maidenhead, Berks.

2.6.2. Chromatographic techniques.

2.6.2.1. Gel filtration chromatography.

Gel filtration chromatography separates molecules according to their molecular size and shape. In this work the procedure was mainly used for the fractionation of serum proteins and of SE. Three gel filtration media were used, Sephadex G200, Sepharose 6B and Sepharose 4B (Pharmacia (GB) Ltd., London).

Sephadex G200 has an appropriate fractionation range in molecular weight (MW) of 5,000 - 800,000 for peptides and globular proteins and 1,000 - 200,000 for dextrans or linear polysaccarides. Sepharose 6B has exclusion limits of $4x10^6$ for globular proteins and $1x10^6$ for linear polysaccarides, whereas Sepharose 4B has exclusion limits of $20x10^6$ for globular proteins and $5x10^6$ for linear polysaccarides.

Gel filtration chromatography was carried out according to the procedures outlined by Pharmacia (1971). The Sephadex G200, Sepharose 6B or Sepharose 4B were packed into Pharmacia K 26/100 (Pharmacia (G.B.) Ltd.) columns which were 2.6 cm in diameter and 100 cm long.

Figure 2 outlines the equipment layout for gel filtration. Samples and buffer were pumped onto the columns with upward flow using either an LKB Varioperpex peristaltic pump 12,000 (LKB Instruments Ltd., South Croyden, Surrey) or a

Pharmacia peristaltic pump P3 (Pharmacia (GB) Ltd.). The flow rate used for Sephadex G200 was 3.4 ml/cm^2/hr. Sepharose 6B was used at a flow rate of 4.9 ml/cm^2/hr and Sepharose 4B at 3.8 - 4.5 ml/cm^2/hr. A 0.1M Tris/HCl buffer pH 8.0, containing molar NaCl and 0.002 per cent NaN_3 was used routinely as the elution buffer.

From the column the effluent could optionally be passed through an Amicon CEC1 column effluent concentrator containing a Diaflow Ultrafiltration Membrane of a nominal MW cut off of 10,000 (Amicon Corporation, Lexington, Mass., U.S.A.) to give a concentration of up to five fold. The effluent was passed through an LKB Uvicord Optical Unit 4,700 (LKB Instruments Ltd.) to measure the percentage transmission at 254 nm, which was recorded by an LKB Recorder Unit Type 6,520-4 (LKB Instruments Ltd.). Finally the effluent was collected on an LKB Ultrorac Fraction Collector 7,000 (LKB Instruments Ltd.).

Alternatively, the optical density of the effluent could be monitored at 220 nm by pumping it through a 0.2 mm flow cell in a Pye Unicam SP 600 spectrophotometer (Pye Unicam Ltd., Cambridge) linked to a DC voltage amplifier with a gain of up to x 40. This apparatus enabled the voltage from the SP 600 to register on the LKB recorder unit.

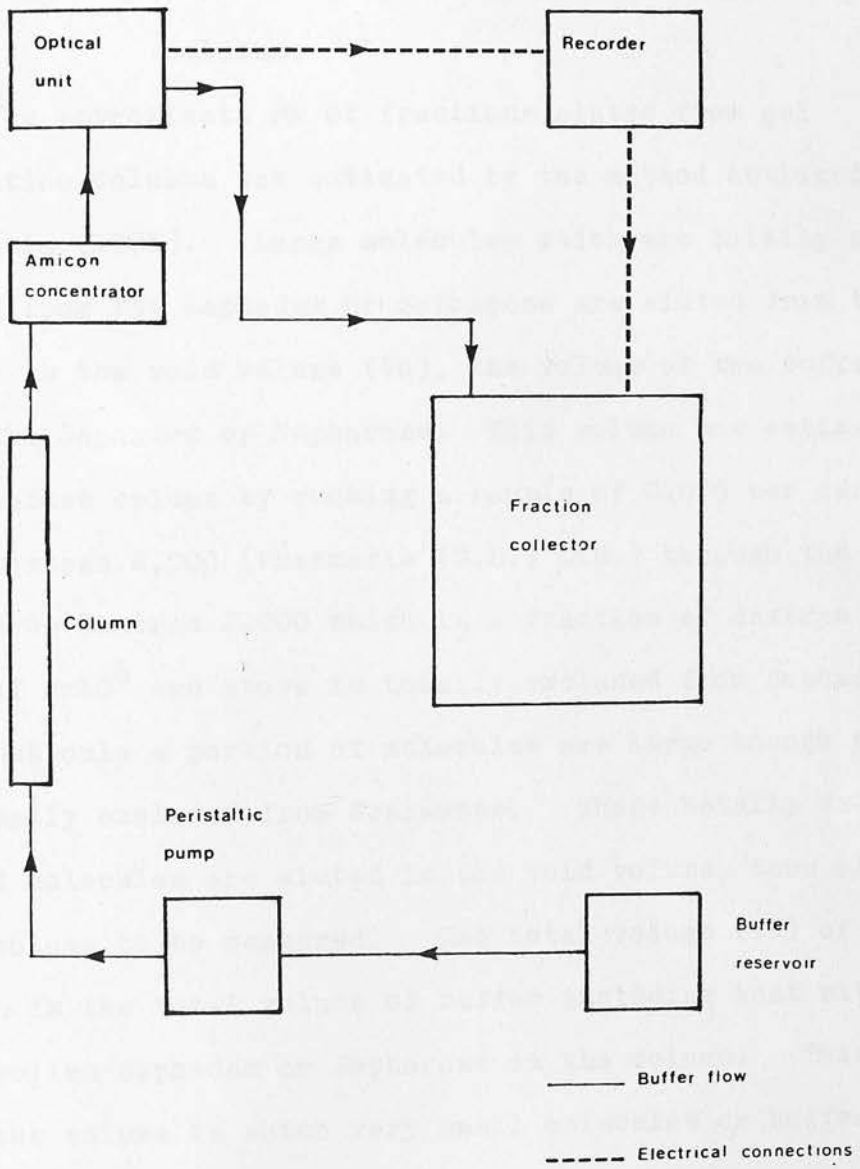


Figure 2. The equipment set up for gel filtration chromatography.

2.6.2.1.1. Determination of the molecular weight of fractions eluted from gel filtration columns.

The approximate MW of fractions eluted from gel filtration columns was estimated by the method outlined by Pharmacia (1971). Large molecules which are totally excluded from the Sephadex or Sepharose are eluted from the column in the void volume (Vo), the volume of the buffer outside the Sephadex or Sepharose. This volume was estimated for each packed column by running a sample of 0.075 per cent Blue Dextran 2,000 (Pharmacia (G.B.) Ltd.) through the column.

Blue Dextran 2,000 which is a fraction of dextran with a MW of $2x10^6$ and above is totally excluded from Sephadex G200 but only a portion of molecules are large enough to be totally excluded from Sepharose. These totally excluded molecules are eluted in the void volume, thus allowing this volume to be measured. The total volume (Vt) of the column is the total volume of buffer including that within the swollen Sephadex or Sepharose in the column. This is also the volume in which very small molecules or buffer ions are eluted from the column.

Sample molecules of intermediate size are eluted from the column mainly according to their molecular size. However, as the true relationship between the molecular weight and elution volume is different for different shapes of molecules i.e. globular proteins or linear polysaccarides a separate calibration curve has to be determined for each

type of molecule.

Since the exact composition of the molecules in SE was not known, the possible MW for each fraction was estimated for both globular and linear molecules using the formula

$$K_{av} = \frac{V_s - V_o}{V_t - V_o}$$

Where V_s = sample elution volume

V_o = void volume

V_t = total volume

and the published selectivity curves (Pharmacia), of K_{av} against molecular weight.

2.6.2.2. Ion exchange chromatography.

Ion exchange chromatography was used for the fractionation of serum proteins and of SE. Diethylaminoethyl cellulose (DE52) and carboxymethyl cellulose (CM52) (Whatman Laboratory Sales Ltd., Maidstone, Kent) preswollen microgranular ion exchange celluloses were used. DE cellulose is an anion exchanger whereas CM cellulose is a cation exchanger.

Before use the celluloses were degassed, equilibrated and the fines removed according to the methods outlined by the manufacturer (Whatman Technical Bulletin IE2). When equilibrated to the starting buffer, the pH and conductivity of the buffer eluted from the cellulose was identical to that added to it. The pH was measured on a Pye Unicam pH Meter (Pye Unicam Ltd.) and the conductivity on a Phillips Laboratory Conductivity Meter

(Phillips Ltd., Eindhoven, Holland).

2.6.2.2.1. Elution of samples from ion exchange celluloses.

There are two main procedures used in handling ion exchange celluloses, namely either batchwise separation or the ion exchange cellulose is packed into a chromatographic column and the samples eluted either by stepwise or continuous gradient elution.

2.6.2.2.1.1. Batchwise separation of SE.

This procedure involves adding a known quantity of equilibrated and fines free ion exchanger to the sample. The two are then stirred together to allow the sample to become adsorbed to the cellulose. The ion exchanger is separated from the exchanger by centrifugation and is then washed with the buffer used for equilibration. Fractions of the sample can then be eluted from the cellulose by changing the elution conditions. This procedure was used in the preliminary work on the saline extract of T. saginata to find the most suitable ion exchanger for use with this extract.

Five g of DE52 were equilibrated to 0.01 M Tris/HCl buffer, pH 7.2 and 5 g of CM52 were equilibrated to 0.01 M sodium phosphate buffer, pH 7.2. Following equilibration the celluloses were centrifuged at 1,500 g for 5 minutes and the supernatants discarded. Two 1 g aliquots of both DE52 and CM52 were weighed out and placed in test tubes.

Each of the 4 aliquots were mixed with 2 ml of SE for 30 minutes at room temperature. The tubes were centrifuged at 1,500 g for 5 minutes at room temperature and the supernatants retained for analysis (DES1 and CMS1). The celluloses were resuspended in 5 ml of the appropriate starting buffer and centrifuged at 1,500 g for 5 minutes at room temperature. The washing process was repeated twice more and the supernatants discarded. After the final wash 2 ml of eluting buffer of either acid or alkaline pH and high molarity was added to the cellulose and the two mixed at room temperature for 30 minutes. The test tubes were again centrifuged at 1,500 g for 5 minutes and the supernatants, following dialysis against PBS pH 7.3 were retained for analysis, giving CMS2, CMS3, DES2 and DES3.

The elution buffers for CM52 and 0.1 M sodium acetate/ acetic acid buffer, pH 5.0 containing 1 M NaCl and 0.1M sodium borate / NaOH buffer, pH 9.0 containing 1 M NaCl and for DE52 were 0.1 M sodium acetate / acetic acid buffer, pH 5.0 containing 1 M NaCl and 0.1 M Tris / HCl buffer, pH 8.9 containing 1 M NaCl.

On analysis the supernatants were checked for HI activity by the HI technique and for gel precipitins by the micro gel precipitation technique. Protein estimations were made by the Folin technique and the carbohydrate content was estimated by the anthrone reaction.

2.6.2.2.1.2. Column procedures.

The equipment layout for ion exchange chromatography

is detailed on Figure 3. The cellulose was packed into Whatman glass columns 2.5 cm in diameter (Whatman Laboratory Sales Ltd.). Samples and buffer were pumped through the column with upward flow usually at a flow rate of 50 ml / hr (10.2 ml /cm^2 /hr).

The procedure for monitoring the column effluents and collecting the fractions was the same as that outlined for gel filtration chromatography (2.6.2.1.). Samples were pumped onto the column at a pH and molarity at which the desired substances were adsorbed onto the cellulose. Two different methods were used to elute samples from the ion exchange columns, namely either stepwise or gradient elution.

2.6.2.2.1.2.1. Stepwise elution.

A pre-selected buffer was pumped onto the column and a fraction eluted which was then collected. When one fraction was completely eluted the buffer was changed and another buffer pumped onto the column which would elute a second fraction and so on. This technique was used to purify serum proteins.

2.6.2.2.1.2.2. Gradient elution.

In gradient elution a constantly increasing molarity of eluting buffer was produced by mixing two buffers, one of high molarity and one of low molarity, together. The rate of change in the strength of the eluting buffer and the time taken for a complete chromatographic run was

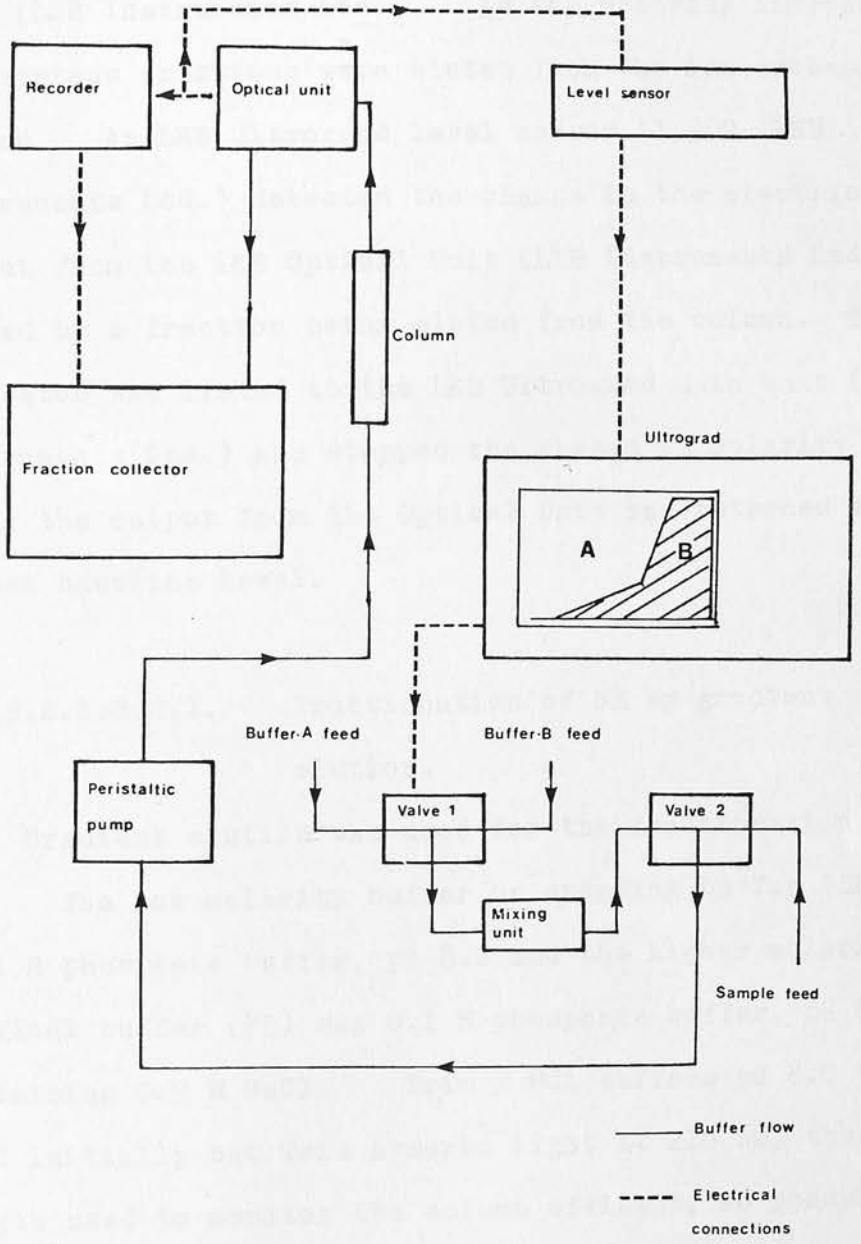


Figure 3. The equipment set up for ion exchange chromatography – gradient elution

controlled automatically using LKB Ultrograd 113,000 equipment (LKB Instruments Ltd.). As the molarity increased, the various fractions were eluted from the ion exchange column. An LKB Ultrograd level sensor 11,300 (LKB Instruments Ltd.) detected the change in the electrical output from the LKB Optical Unit (LKB Instruments Ltd.) caused by a fraction being eluted from the column. This apparatus was linked to the LKB Ultrograd main unit (LKB Instruments Ltd.) and stopped the change in molarity until the output from the Optical Unit had returned to a preset baseline level.

2.6.2.2.1.2.2.1. Fractionation of SE by gradient elution.

Gradient elution was used for the fractionation of SE. The low molarity buffer or starting buffer (SB) was 0.01 M phosphate buffer, pH 8.0 and the higher molarity or final buffer (FB) was 0.1 M phosphate buffer, pH 8.0 containing 0.9 M NaCl. Tris / HCl buffers pH 8.0 were used initially but Tris absorbs light at 220 nm, the wavelength used to monitor the column effluent, so phosphate buffers were used instead. This unfortunately meant that an anionic buffer was being used with an anion exchanger which is theoretically suboptimal, however, phosphate buffers have often been used with anion exchangers such as DE cellulose e.g. Penhale & Christie (1969).

2.6.2.3. Immunoadsorption columns.

Immunoadsorption columns were prepared by first mixing a volume of immunoadsorbent with an equal volume of fibrous cellulose powder (CF1; Whatman Laboratory Sales Ltd.) from which the fines had been removed. The mixed cellulose and immunoadsorbent was packed into a 1 cm diameter Whatman column (Whatman Laboratory Sales Ltd.) at a flow rate of 50 ml/hour (63.7 ml /cm^2 /hr by the same method used for DE cellulose (2.6.2.2.).

The cellulose provided an inert matrix throughout the length of the column. The immunoadsorbent was distributed around the fibres, which reduced the resistance to flow of the buffer and prevented the column from compacting.

Before use the columns were washed for some time with PBS pH 7.3 and the absorption of the effluent monitored at 254 nm. The elution buffer to be used, usually 0.1 M glycine / HCl buffer, pH 2.8 containing 2.5 M NaCl was then pumped onto the column. Any loosely bound material was eluted in one large peak. The column was washed again with PBS pH 7.3 and the procedure repeated once more to ensure that all the loosely bound material had been eluted.

Following an overnight wash with PBS pH 7.3, the column was ready for use. After use, the column was washed with PBS pH 7.3 containing 0.02 per cent NaN_3 and stored with this solution to prevent bacterial contamination.

2.6.2.3.1. Fraction of SE on immunoadsorption columns.

Two immunoadsorbents were used for this experiment, one was prepared from a pool of whole normal bovine serum (N14 and N15) and the other was part of the immunoadsorbent prepared from the pooled serum from cattle experimentally infected with T. saginata eggs (2.6.1.9.2.). The serum for this immunoadsorbent had been precipitated with 40 per cent ammonium sulphate.

An appropriate volume of SE was dialysed overnight versus PBS pH 7.3 at 4°C. The dialysed sample was pumped onto the column, containing the normal bovine serum immunoadsorbent, at 25 ml/hr (31.8 ml/cm^2/hr.). The non adsorbed material (F1), eluted with the PBS ph 7.3 washing buffer, was collected. Following a further wash with PBS pH 7.3 any adsorbed material (F2) was eluted with 0.1 M glycine / HCl buffer, pH 2.8 containing 2.5 M NaCl. This fraction was collected and dialysed extensively against PBS pH 7.3.

The non adsorbed components of SE (F1) were then applied to the column containing the immunoadsorbent prepared from the sera of T. saginata infected calves. The flow rate was again 25 ml / hr (31.8 ml/cm^2/hr). The non adsorbed material (F3) was eluted with the PBS pH 7.3 washing buffer and collected. Following a further wash with PBS pH 7.3 any adsorbed material (F4) was eluted with 0.1 M glycine / HCl buffer, pH 2.8 containing 2.5 M NaCl. This fraction was dialysed extensively against PBS pH 7.3.

Fractions F2, F3 and F4 were all filtered and concentrated to twice the original sample volume by dialysis against carbowax. The fractions were stored at - 20°C until use.

2.7. Assay procedures.

2.7.1. The Folin phenol technique.

The method used was after that of Lowry, Rosebrough, Farr & Randall (1951). The technique measures mainly the tryptophan and tyrosine content in a sample, hence giving an estimate of the protein content.

Reagents -

Solution A 2 per cent Na_2CO_3 in 0.1 M NaOH.

Solution B 0.5 per cent $CuSO_4 . 5H_2O$ in 1 per cent sodium tartrate.

Solution C 50 ml of solution A was mixed with 1 ml of solution B. This solution was renewed daily.

Solution D Folin and Ciocalteau's phenol reagent (BDH Chemicals Ltd.) diluted 1:3 with distilled water.

For protein samples containing 25 - 500 µg of protein in 1 ml, 5 ml of solution C was added, mixed well and allowed to stand for 10 minutes at room temperature. Then 0.5 ml of solution D was added and mixed immediately. The reagents were allowed to stand for 30 minutes before the absorbance was read using an EEL Portable Calorimeter (Evans Electroselenium Ltd., Halstead, Essex), and an

Ilford 608 filter (maximum transmission 608 nm). A distilled water blank was included as a control.

A calibration graph was prepared by making known dilutions of a serum standard, Versatol (W. R. Warner & Co. Ltd., Eastleigh, Hampshire) of up to 5 g / 100 ml (g per cent) The protein content of a sample was estimated by comparison with the calibration graph and the results expressed in µg.

2.7.2. The biuret technique.

The biuret technique in routine use was a modification of that of Henry, Sobel & Berkman (1957). The technique measures mainly the peptide linkages in proteins and hence gives an estimate of the protein content of a sample.

Reagents -

Biuret reagent was made by dissolving 8.65g of $CuSO_4 . 5H_2O$ in 50 ml of hot distilled water and 86.5 g of sodium citrate plus 50 g of Na_2CO_3 (anhydrous) in a further 200 ml of hot distilled water. When the solutions were cool the copper sulphate solution was poured into the latter solution and made up to 500 ml with distilled water.

The following reagents were placed in a 7 ml Wasserman tube - 0.1 ml of serum, distilled water or a dilution of standard, plus 3.9 ml of 3 per cent NaOH solution and 1 ml of biuret solution. The solutions were mixed well and left for 15 - 30 minutes. The absorbance was read on an EEl Portable Colorimeter (Evans Electroselenium Ltd.) against a distilled water blank, using an Ilford 545

filter (maximum transmission 545 nm).

A calibration graph was prepared by making standard dilutions of Versatol (W.R. Warner Co. Ltd.) containing up to 150 g/100 ml (g per cent). The protein content of a sample was estimated by comparison with the calibration graph and the results expressed as g per cent.

2.7.3. Protein estimation by measuring the extinction at 260 nm and 280 nm.

This method was developed by Warburg & Christian (1941). The technique depends mainly on the phenylalanine, tryptophane and tyrosine content of the sample and does not measure total protein. This technique is, however, particularly useful because of the ease and rapidity of handling the samples and because little or no sample is lost in the test.

The extinction of an appropriately diluted protein solution was measured at both 260 nm and 280 nm on a Pye Unicam spectrophotometer SP 600 (Pye Unicam Ltd.). The ratio E280 : E260 was calculated. Using this ratio, the proportion of nucleic acids in the protein sample and a factor for calculating the protein content could be read off from a standard table (Warburg & Christian, 1941). The protein concentration was estimated using the following formula:-

Protein concentration mg/ml = E280 nm x F x $\frac{1}{10}$ x D

Where F = the factor mentioned above

b = the length of the light path

D = dilution

The figures in the table Warburg & Christian, (1941) were calculated from the extinctions of crystaline yeast enolase (E260 nm = 1.18, E280 nm = 2.06 for solutions containing 1 mg/ml). The method is liable to a proportionate error in so far as other proteins and nucleic acids have different extinctions.

2.7.4. Carbohydrate estimation - the anthrone reaction.

The amount of carbohydrate in antigen samples was estimated by the anthrone reaction using the hexose sugar glucose as a standard. The method was after that of Yemm & Willis (1954).

Anthrone reagent was prepared as described by Trevelyn & Harrison (1952) by dissolving 0.2 g of anthrone (BDH Chemicals Ltd.) in 100 ml of H_2SO_4 (made by dissolving 500 ml of concentrated acid in 200 ml of distilled water). The reagent was allowed to stand for 30 - 40 minutes with occasional shaking until it was perfectly clear. The reagent was freshly prepared each day and used within 12 hours.

The reaction was carried out under conditions similar to those used by Trevelyan & Harrison (1952). The anthrone reagent (5 ml) was pipetted into 1.5 cm x 15 cm pyrex tubes and chilled in ice water. The solution

under test (1 ml) was layered onto the anthrone reagent, cooled for a further 5 minutes and then thoroughly mixed while the tube was still immersed in ice water. A glass sphere was placed on the top of each tube to prevent evaporation. The tubes were incubated in a boiling water bath for 10 minutes then cooled for 5 minutes in ice water.

The absorbance of solutions were read on an EEl Portable Colorimeter (Evans Electroselenium Ltd.), using an Ilford 607 orange filter (maximum transmission 607 nm). The measurements of the test solutions, standards and reagent blanks were made against distilled water as a reference. A calibration graph was prepared each day by making standard dilutions containing 20 - 100 µg of glucose / ml. A saline control was also included. The amount of hexose sugar in any sample was estimated by comparison with the calibration graph. The results were expressed as µg / ml.

2.7.5. The tanned cell indirect haemagglutination (IDH) technique.

At the beginning of this work the IDH technique as developed by Boyden (1951) was already in routine use for the detection of haemagglutinating antibodies in the sera of cattle experimentally infected with T. saginata (Gallie & Sewell, 1974 a+b). The technique being used was basically that of Boyden (1951) and was carried out in MRC plates. However, for this study the technique

was performed on a micro scale (Figure 4) in order to reduce the amounts of reagents and the time taken to carry out the procedure.

The equipment for such a micro technique was described by Sever (1962). It is available commercially (Titretek Microtitration Equipment, Flow Laboratories Ltd., Irvine, Scotland) and consists of small plastic disposable plates each with 96 wells (Titretek/Linbro Plates with V shaped wells, Flow Laboratories Ltd.), which will hold up to 0.3 ml, droppers calibrated to deliver 0.025 ml drops of solution and microdiluters which are calibrated to pick up 0.025 ml volumes.

2.7.5.1. Preparation of red blood cells and serum for the IDH technique.

Sheep red blood cells (rbc's) were prepared as described by Campbell et al (1964). A 1.5 ml volume of sheep rbc's in Alsever's solution (2.4.3.) was placed in a conical test tube and made up to 10 ml with 5 per cent BBS. The 5 per cent BBS was made by adding 50 ml of borate buffer stock (2.6.1.2.) to 950 ml of 0.85 per cent saline solution. The suspension was centrifuged at 1,500 g for 5 minutes and the supernatant discarded. The washing process was repeated three times. After the final centrifugation the rbc's (equivalent to about 0.25 ml of packed cells) were made up to 10 ml in PBS pH 7.3.

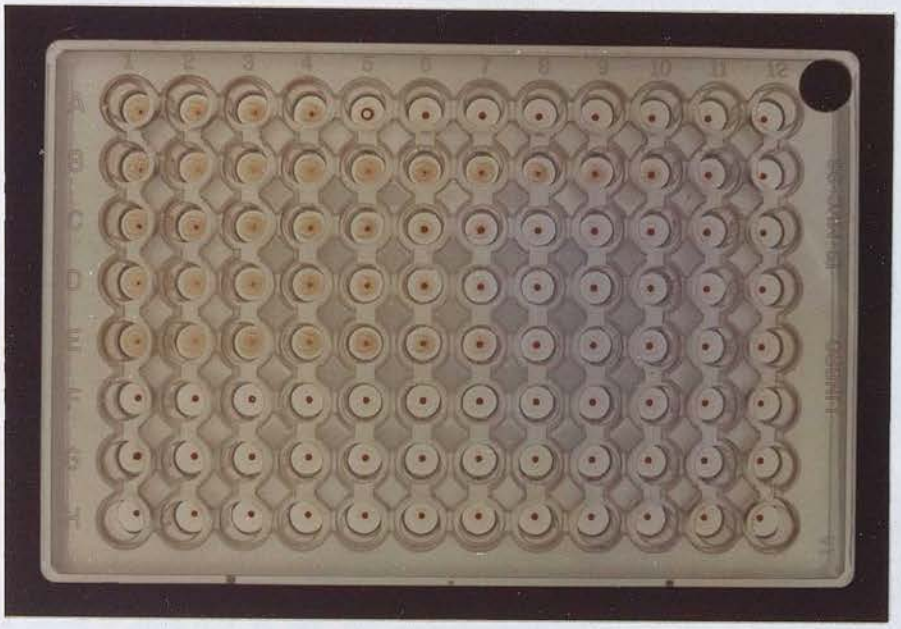


Figure 4. A micro haemagglutination (IDH) test for cattle infected with T. saginata cysticerci, showing haemagglutination with five positive sera (rows A-E) and a negative reaction with three normal control sera (rows F-H).

The cells were tanned by adding 3 ml of washed sheep rbc's to an equal volume of 0.005 per cent tannic acid solution in 0.85 per cent NaCl and incubating the solution at 37°C for 15 minutes. Following centrifugation at 1,500 g for 5 minutes. The supernatant was discarded and the rbc's resuspended in 3 ml of PBS pH 7.3. The washing procedure was repeated once more and the rbc's resuspended in PBS pH 7.3.

The optimum concentration of antigen for use in the IDH technique was determined by coating washed and tanned sheep rbc's with various concentrations of antigen. An IDH test was then set up for each group of cells using standard positive and normal sera. The optimum concentration of antigen gave the maximum IDH titres, this usually corresponded to about 5 µg of antigen protein / ml.

To coat the tanned rbc's in antigen, 1 ml of antigen solution diluted to optimum concentration in PBS pH 6.4 (made from 100 ml of buffer consisting of 32.5 ml of 0.15 M Na_2HPO_4 and 67.8 ml of 0.15 M KH_2PO_4 plus 100 ml of normal saline solution) was added to 1 ml of tanned rbc's and 4 ml of PBS pH 6.4. The mixed solutions were incubated at room temperature for 15 minutes, centrifuged for 5 minutes at 1,500 g and the supernatant discarded. The rbc's were resuspended in 3 ml of diluent (5 per cent BBS containing 1 per cent inactivated and absorbed (I+A) normal bovine serum). The presence of the I+A bovine serum prevented pan-agglutination.

Directly prior to use the rbc's were again centrifuged, and the precipitated cells made up to 10 ml

with diluent. This gave a 0.25 per cent suspension of rbc's. In the macro test the red blood cells were used at a concentration of 2.5 per cent. A 0.05 ml drop of a 2.5 per cent rbc suspension was added to 0.5 ml of (I+A) serum in the walls of the MRC plates. In the micro /

test the equivalent amount of rbc's were added to the 0.025 ml of serum in the wells by adding one 0.025 ml drop of a 0.25 per cent suspension of rbc's.

Prior to use in the IDH test all sera were first inactivated by incubation in a water bath at 56°C for 30 minutes to destroy the complement. They were then absorbed with 3 ml of the washed sheep rbc's suspended in PBS pH 7.3, for 30 minutes at room temperature to remove antisheep antibodies present in the bovine serum. The supernatant was retained after centrifugation for 5 minutes at 1,500 g and the precipitate discarded. The (I+A) sera were stored at -20°C until use. Due to the addition of the rbc solution the sera were now diluted 1:2.

2.7.5.2. Preparation of microdiluters and microtitre plates.

As stated previously a micro system was used for this technique. The microdiluters picked up 0.025 ml of solution and were prepared for use according to the manufacturers instructions. Each was flamed to dull red luminescence, then quenched in distilled water. Directly prior to use each one was checked for delivery by dipping it in 0.85 per cent saline then placing it on the maker's delivery testing card (marked blotting paper). If a microdiluter was functioning correctly the solution it carried quickly came off and onto the card, marking a standardised area.

A 0.025 ml drop of diluent was placed into each well

of a microtitre tray using a calibrated 0.025 ml dropper from the microtitre system. A 0.025 ml quantity of the (I+A) serum to be tested was picked up by a prepared microdiluter which was placed in the first well in the plate. The microdiluter was gently rotated in the well to mix the serum with the diluent, then placed in the next well of the row. The solutions were mixed and so on until by repeating this process a serial dilution was made. The last well of any row was always left with no test serum in it as a diluent control. Next, a 0.025 ml drop of prepared rbc's at a concentration of 0.25 per cent was added to each well and the plate gently tapped to mix the serum and the cells. The plates were left overnight at $4^{o}C$ for a pattern to develop. This pattern, produced by the settled cells, remained stable for several days if the plates were kept at $4^{o}C$.

2.7.5.3. The controls for the IDH technique and reading the results.

The controls used for the IDH technique are summarised in Table 2. Only the TAg+ cells with positive serum should haemagglutinate, all the other control titrations should be negative, as should the diluent control included in each row. However, as these were almost always negative only two of the controls were set up routinely, these being TAg+ cells with the standard positive and control sera. In addition each row included a diluent control. The inclusion of these controls with each test also allowed a day to day check to be made on the titres.

Table 2 Controls for use with the IDH technique.

Type of cells added	Serum in serial dilution in plate	
*TAg+	Positive serum	Test
TAg-	"	Control
NTAg+	"	"
NTAg-	"	"
*TAg+	Normal control serum	Control
TAg-	"	"
NTAg+	"	"
NTAg-	"	"

T = tanned rbc's

NT = not tanned rbc's

Ag+ = cells coated in antigen

Ag- = cells not coated in antigen

i.e. TAg+ = Tanned cells coated in antigen.

* Controls used in all studies.

Reading the results of the IDH technique is always somewhat subjective and this is one of the major disadvantages of the technique. The following method of scoring the plates was selected.

++ positive - a smooth mat of cells at the bottom of the well.

+ negative - the cells beginning to pull away from the well.

+- negative - a small button beginning to appear at the bottom of the well.

-+ negative - most of the cells forming a button at the bottom of the well but still some haemagglutination.

- negative - all the cells forming a button at the bottom of the well - no haemagglutination.

The particular point that is taken as the end point or titre for the titration is quite arbritrary. Different workers have selected different points. Thus Grossklaus and Walther (1972) selected as the titre the dilution in the last well with any haemagglutination at all, whereas Gallie & Sewell (1974 a+b) took the titre as the dilution in the last well with a + reading. In this study the serum dilution in the last well with a ++ reading was taken as the titre for that serum. It was considered that this would lead to less variation from day to day.

It is inevitable that in a technique such as this, with many stages, there will be minor differences occurring from day to day in the details of the technique and this can lead to a variation in the titre of as much as + or - one doubling dilution. The variation in a ++ reading will be less than that using the lower + or -+ end point because of the relatively large amount of antiserum required

for a ++ reading.

Weak serum samples from the field were sometimes not strong enough to give a ++ reading. However, the presence of antibody could still be detected by noting the last well with any haemagglutination at all as is routinely done by Walther & Grossklaus (1972).

2.7.6. The haemagglutination inhibition (HI) technique.

Boyden (1951) pointed out that the addition of a given antigen to its homologous antiserum resulted in the inhibition of the effect of the latter in agglutinating erythrocytes sensitised with this antigen, and that this effect was specific. The degree to which a given antigen will inhibit a homologous antiserum from agglutinating erythrocytes sensitised with this antigen can be used as a measure of the concentration of the antigen in a solution. Read & Bryan (1960) described the principal behind the HI technique (Figure 5).

An aliquot of a solution which may or may not contain antigen is added to an equal aliquot of antiserum, the latter being at a concentration greater than at its end point in the IDH technique. To the mixture is then added tanned, antigen coated rbc's. If there was no or very little antigen in the first solution, haemagglutination will occur (Figure 5.1). However, if there was sufficient antigen present to neutralise all the antibody in the antiserum, no haemagglutination will occur (Figure 5.2.)

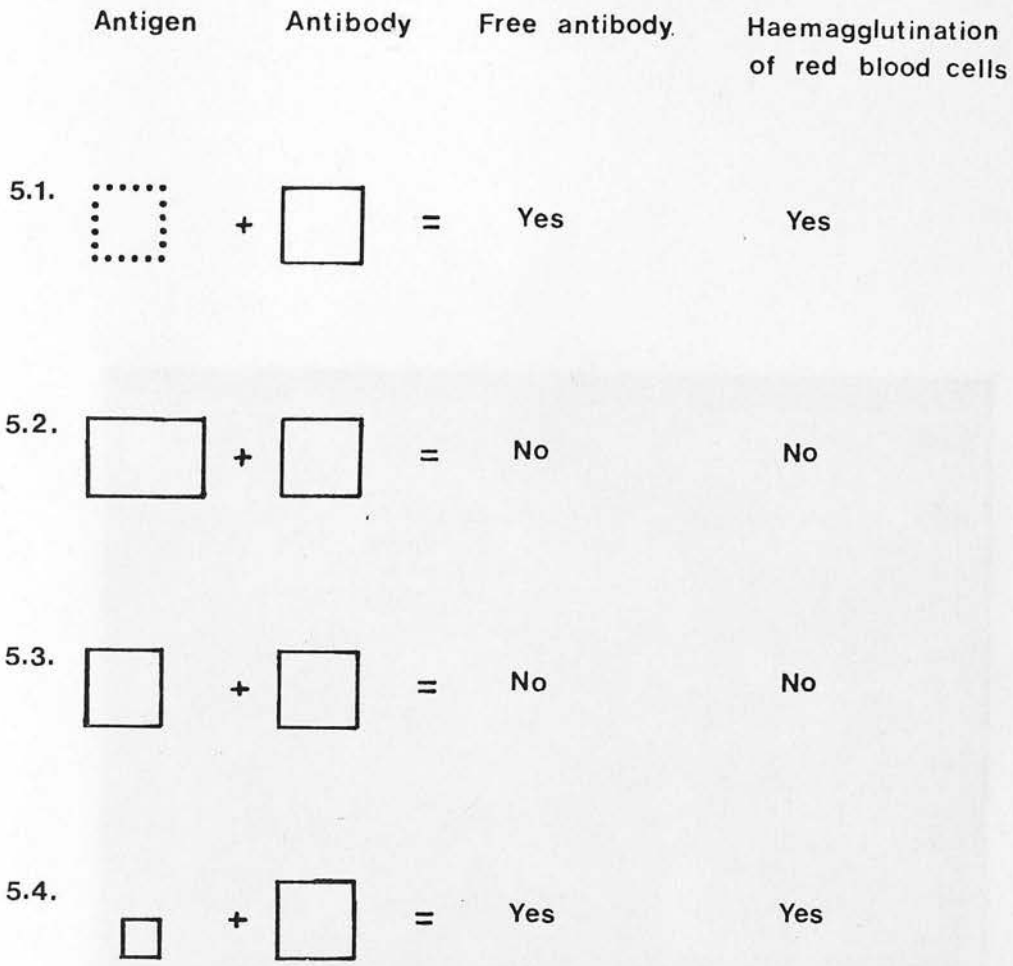


Figure 5. A schematic representation of the principal of the HI technique

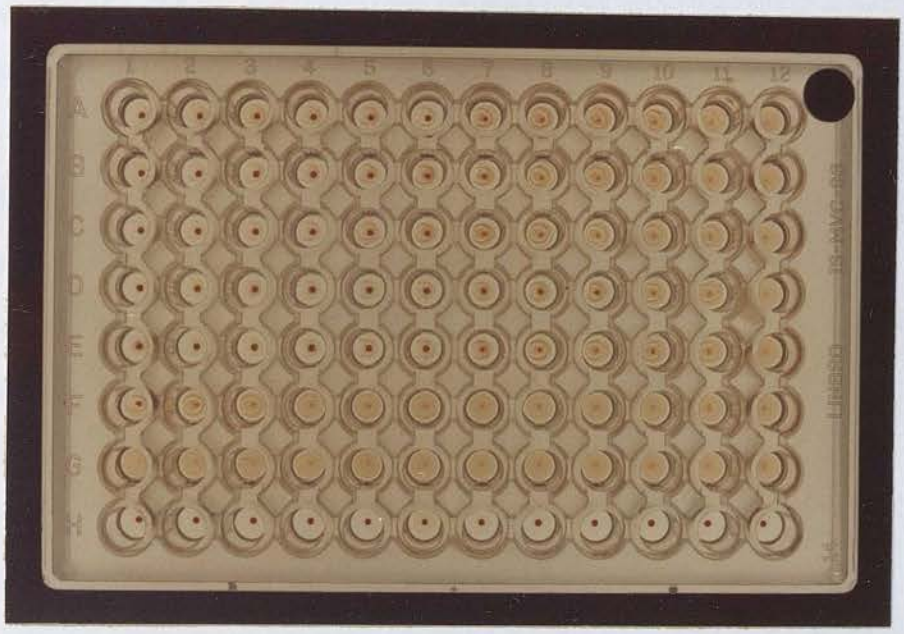


Figure 6. A micro haemagglutination inhibition (HI) test for T. saginata antigen, showing inhibition of haemagglutination with six T. saginata antigen samples (rows A-F). Saline controls are in row G and diluent controls are in row H.

Therefore, when decreasing concentrations of antigen are added to successive wells or tubes containing the same concentration of antiserum, a point is eventually passed when all the antibody is neutralised (Figure 5.3.), and the remaining free antibody (Figure 5.4.), causes haemagglutination of the tanned and antigen coated cells. At first such haemagglutination is slight (Figure 6) but complete haemagglutination is seen in the wells containing sufficiently high dilutions of the antigen solution.

The procedure followed for the HI technique was similar in principal to that outlined by Sever (1962). The method used to tan and coat the rbc's in antigen was identical to that used in the IDH technique (2.7.5.1.), except that the cells were used at a concentration of 0.5 per cent rather than the 0.25 per cent used in the IDH technique. This increased concentration of cells facilitated reading the plates.

First 0.025 ml drops of diluent were placed in the wells of a microtitre plate using a calibrated dropper. A serial dilution of the antigen under test was made in a row of wells in the same manner as that described for serum in the IDH technique. To this diluted antigen was added one 0.025 ml drop of standard positive serum from a calf orally infected with T. saginata. The serum was diluted to contain 4 haemagglutination units with one unit defined as the ++ titre for the serum. The solutions were mixed by gently tapping the plate which was then left at room temperature for

30 minutes. A 0.025 ml drop of 0.5 per cent tanned and antigen coated rbc's was then added and the plate gently tapped to mix the cells with the solution. The plates were left overnight at 4°C for the pattern to develop, and the results read next morning.

The same criteria were used for scoring the HI plates as that used for the IDH technique. As with Sever (1962) the end point or titre for an antigen sample was considered to be the last well to show complete inhibition of haemagglutination. This end point was selected as it was easier to determine the last well with no haemagglutination than the first well with complete haemagglutination, the method adopted by Boyden (1951) and Read & Bryan (1960).

The controls routinely incorporated into the IDH technique were included for the HI technique since this technique also uses tanned and antigen coated rbc's. These were TAg+ cells with positive and normal control sera and a diluent control. The standard positive serum diluted to contain 4 haemagglutination units was tested to check that the diluted serum would cause haemagglutination of the tanned and antigen coated rbc's. A serial dilution of the antigen without any antiserum added was included to ensure that antigen alone did not cause the haemagglutination of the rbc's. As well as the test antigen samples, a standard antigen was included in each test to check for day to day variation.

2.7.7. The micro double diffusion (MGP) technique.

At the beginning of this work the MGP technique was already in routine use for the detection of precipitating antibodies in the sera of cattle experimentally infected with T. saginata (Gallie & Sewell, 1974 a+b). The procedure followed was a slight modification of that of Crowle (1958). This technique, first described by Wadsworth (1957), uses plastic templates with holes drilled in them for feeding relatively large quantities of reactants into a small cross sectional area of agar gel, so providing a miniaturised double diffusion test, which is sensitive when compared to other double diffusion techniques.

Glass slides 76x25 mm (Chance Proper Ltd., Worcester) were steeped in a solution of Decon 75 (Decon Laboratories Ltd., Brighton) for 30 minutes, rinsed several times in distilled water and dried in an incubator at 37°C. The clean dry slides were polished to remove any marks, flamed, dipped into a boiling aqueous solution of 0.02 per cent purified agar (Oxoid Ltd.) containing 0.001 per cent NaN_3, then left to dry in a slide rack.

A row of agar coated slides was placed in a flat cardboard slide holder and three layers of 2 cm wide plastic tape (RS Components Ltd., London) were stuck across the slides in two parallel rows, 2 cm apart (Figure 7). The slide holder was marked with two central lines 2 cm apart to guide the positioning of the plastic tape. The taped

slides were transferred to a wooden board and cut apart with a scalpel blade.

The plastic templates were made from 2.5 cm squares of perspex, 3mm thick and the wells formed by first boring a 1.5 mm diameter hole through the template. The top was then widened to three quarters of the depth of the template with a 3 mm diameter drill, thus forming a funnel shape. Two patterns of wells were used (Figure 7) the first had 4 wells placed 5 mm from the central well to form a square and the second had 6 wells placed round a central well at a distance of 5 mm to form a hexagon.

The under side of the template was lightly sprayed with silicone grease from an aerosol can (Ambersil Ltd., Basingstoke, Hants) to ensure a good contact between the template and the agar. The silicone grease propellant was allowed to evaporate off before the template was applied to the slide.

Prepared slides were placed on a warm plate at 47°C. Approximately 0.3 ml of 1 per cent purified agar (Oxoid Ltd.), made up in 0.85 per cent saline containing 0.001 per cent NaN_3 and at a temperature of 60°C was pipetted onto the space between the plastic tapes. A silicone coated template was very gently placed on top of the molten agar, siliconised side towards the agar and the template left bridging across the agar, using the plastic tape as support. The film of agar on the slide was approximately 0.5 mm deep.
The slides were removed from the warm plate and allowed to cool.

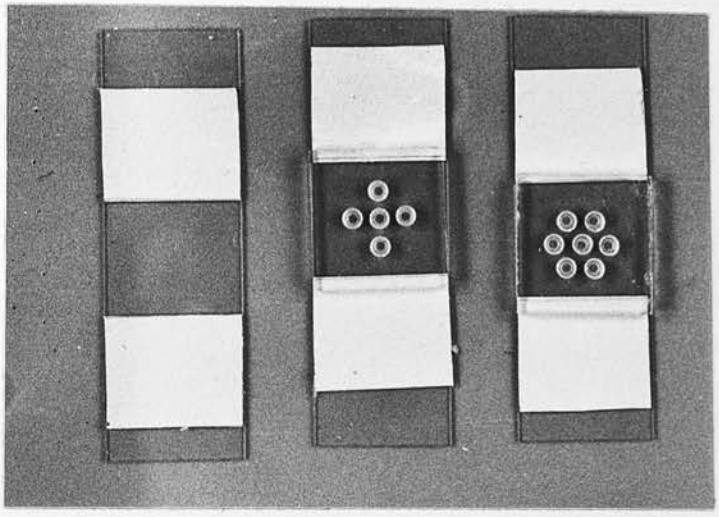


Figure 7. The templates and slides used for the micro gel precipitation technique.

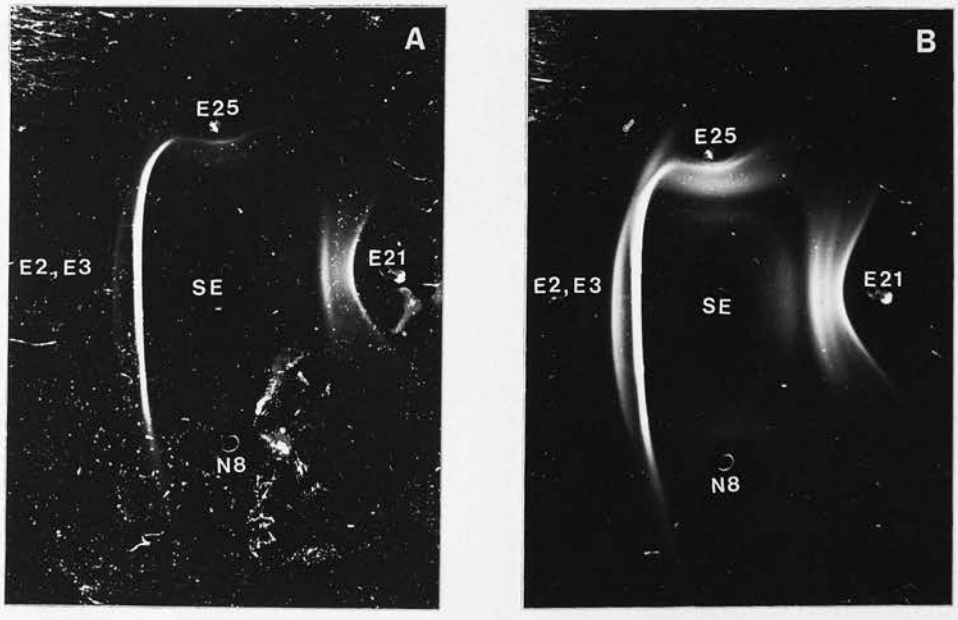


Figure 8. Gel precipitin lines before (a) and after (b) being enhanced by overlaying the gel with rabbit antibovine serum.

Occasionally, some of the prepared template wells were clogged with excess agar, but this could be easily removed with a hypodermic needle with the point bent over to form a tiny scoop. Care had to be taken to avoid dislodging the template from the agar.

Template wells held approximately 0.05 ml of fluid and double drawn Pasteur pipettes were used to fill them with serum or antigen solution, care being taken that the reactant touched the agar and that no air bubble intervened.

The slides plus filled template were placed in plastic boxes which had sheets of damp blotting paper inside the lids to keep the air in the box humid thus preventing the agar from drying out. The lids were sealed in position with plastic tape and the slides left at 22°C for 3 - 4 days to allow the precipitin lines to develop. Templates were removed by applying gentle pressure with the thumb and fore-fingers of both hands until they lifted away from the agar.

The slides were placed in 0.85 per cent saline solution and after about 2 hours the agar was wiped very gently with a dry swab to remove any traces of silicone grease on the surface. Once the silicone was removed the slides were washed in 0.85 per cent saline solution for approximately 24 hours with at least 3 changes of saline solution.

If the precipitin lines were faint they were 'developed' by soaking them in a 0.0125 per cent solution of cadmium acetate (Crowle, 1961), followed by a further wash in 0.85 per cent saline. An alternative technique for developing the precipitin lines was to overlay the agar with a 1:10

dilution of rabbit antibovine serum (Figure 8). The washed slides were placed in a humid chamber and several drops of the antiserum placed on the agar. After 1 hour incubation the slides were washed several times in saline to remove any excess protein prior to staining.
The thin layer of agar on slides was stained with Thiazine Red (George T. Gurr Ltd., London.) using the following schedule, modified from Crowle (1958).

Distilled water wash 1.	10 minutes
" " " 2.	10 minutes
1 per cent Thiazine Red in 1 per cent acetic acid	10 minutes
1 per cent acetic acid wash 1.	10 minutes
" " " " " " 2.	10 minutes
" " " " " " 3.	10 minutes
1 per cent glycerol in 1 per cent acetic acid	10 minutes

The glycerol prevented the slides from cracking. After staining the slides were dried overnight at 37°C. Silicone grease was removed from the templates by boiling them in a 1 per cent solution of Decon 75 (Decon Laboratories Ltd.) for approximately 15 minutes. Following several rinses with distilled water, the templates were finally dried at 37°C. Prior to use the templates were polished with a soft cloth and any scratched templates were discarded as these gave unsatisfactory results.

2.7.8. Assay of precipitating antisera,

Assays of precipitating antisera were carried out by a slight modification of the method described by Beutner, Holbourgh & Johnston (1967). In order to carry out the test, a pattern of six peripheral wells round a central well was cut in 1 per cent purified agar (Oxoid Ltd.) in 0.85 per cent NaCl. The LKB Gelman Immunodiffusion Apparatus (LKB Instruments Ltd.) was used. The wells had a diameter of 2.5 mm and were placed 5 mm apart (centre to centre). Dilutions of antiserum were placed in the outer wells and one well was filled with saline as a control. The central well was filled with a solution of antigen containing 1 mg of protein/ml. After overnight incubation in a humid chamber at room temperature, precipitin lines showing a reaction of identity developed. The titre was considered as the serum dilution where the gel precipitin line met a serum well. Any antiserum with a titre of 1:16 or over was considered acceptable.

2.7.9. Estimation of the appropriate amount of immuno-adsorbent to mix with antiserum.

This test was carried out to obtain a rough estimate of the amount of antiserum or antigen to mix with glutaraldehyde insolubilised antigen or antiserum. Glutaraldehyde insolubilises protein by causing the molecules to clump together to form conglomerates. Only a proportion of the original molecules are still free to react with antiserum or

antigen, the rest are bound up inside the complexes with other globulin molecules. This estimate was, therefore, only approximate.

The test was carried out as described in 2.7.8. with doubling dilutions of the antiserum under test placed in the wells around the central antigen well which contained 1 mg/ml of protein. When the gel precipitin lines developed the dilution of antiserum at which the precipitin line was centred between the wells was taken as the dilution of antiserum which reacted with 1 mg of antigen.

2.7.10. Immunoelectrophoresis.

Immunoelectrophoresis was used to check the purity of the serum samples fractionated on DE cellulose and Sephadex G200. The method followed was slightly modified from Scheidegger (1955). Glass slides 7.6 cm by 5.1 cm were placed on a level platform and 6 ml of 1 per cent purified agar (Oxoid Ltd.) in barbital buffer pH 8.2 and I= 0.1 was pipetted onto the slide. Once the agar was set, a perspex template (Figure 9a) was placed over the slide. The wells were punched out with a 1 mm diameter catheter and the troughs cut with a scalpel blade. A slide could hold up to 4 troughs and 5 wells. The template was then removed and the agar in the wells taken out with a needle. The agar in the troughs was left in position until after electrophoresis. The wells were filled with the sample for analysis and the slides electrophoresed for 90 minutes at 20 v/cm and 4mA/cm using Shandon Electrophoresis

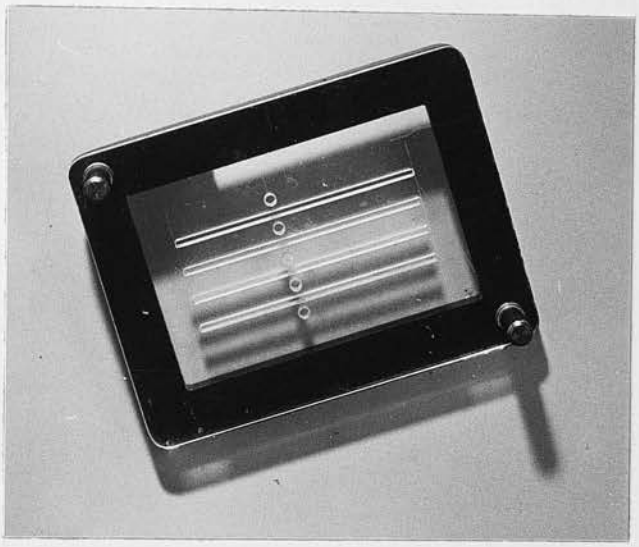


Figure 9a. The perspex template used for immunoelectrophoresis (photographed by kind permission of Dr. A. Luckins).

Figure 9b. The immunoelectrophoresis pattern obtained with whole bovine serum (wells 1 and 5) and 33 per cent (wells 2 and 4) and 40 per cent (well 3) ammonium sulphate fractions of whole bovine serum. Rabbit anti-bovine serum was placed in the troughs.

Apparatus (Shandon Instruments Ltd., Chamberley, Surrey). After electrophoresis the agar in the troughs was removed and they were filled with the appropriate antiserum. The slides were left overnight for the gel precipitin lines to develop. A photograph provided a permanent record (Figure 9b).

2.7.11. Electrophoresis of serum.

Electrophoresis was carried out using a Millipore Clinical Electrophoresis System (Millipore (U.K.) Ltd.). The procedure followed was that outlined by the manufacturers. After electrophoresis the phoroslides were stained and cleared according to the manufacturers instructions and densitometer readings were made on a Millipore Phoroscope Densitometer (Millipore (UK) Ltd.). The proportion of each fraction present in the sample analysed was read off from the scale. A trace was taken to provide a permanent record.

2.7.12. The soluble antigen fluorescent antibody (SAFA) technique.

The SAFA technique was first developed by Toussaint & Anderson (1965) and later further improved (Toussaint, 1966). The technique is essentially similar to the indirect fluorescent antibody technique except that instead of tissue sections on slides being used as antigen, soluble antigen is fixed onto a cellulose acetate matrix.

Figure 10 summarises the procedure involved. Antigen is adsorbed onto cellulose acetate discs. After incubation in an antiserum the discs are washed to remove any excess antiserum before being incubated in a fluoresceinisothiocyanate (FITC) conjugated antiserum. Following a further wash to remove excess FITC conjugate the discs are dried and the degree of fluorescence is measured on a spectrofluorimeter relative to a saline coated control disc.

An advantage of the test is that it can be used with soluble antigen extracts, which can either be crude extracts or those purified by various techniques. In addition the technique overcomes the subjectivity in reading results experienced in serological techniques such as the indirect fluorescent antibody technique, since the degree of fluorescence is measured on a spectrofluorimeter.

The SAFA technique has been used, for example, in detecting the serum antibody levels in parasitic infections such as Trypanasoma cruzi (Toussaint, 1966), human filariasis (Duxbury & Sadun, 1967), amoebiasis (Gore & Sadun, 1968a), trichinosis (Gore & Sadun, 1968b) and E. granulosus and E. multilocularis (Gore & Sadun, 1970).

The procedure was essentially the same as that outlined by Toussaint (1966). Cellulose acetate filter discs 6 mm in diameter, which were gridded on one side were punched from Millipore HAWG 0.45 μ porosity filters (Millipore (UK) Ltd.). The discs were placed with their plain sides in contact with a dilution of the antigen in 0.05 M Tris / HCl

Stage 1.  Antigen (V) attached to cellulose acetate disc (——)

Stage 2.  Addition of antiserum (↔)

Stage 3.  Washing off excess antiserum

Stage 4.  Incubation in FITC conjugate (⋊●)

Stage 5.  Washing off excess conjugate

Stage 6. The degree of fluorescence is measured on a fluorimeter versus a saline coated control disc

Figure 10. A schematic representation of the stages in the soluble antigen fluorescent antibody (SAFA) technique.

buffered 0.85 per cent. saline, pH 8.0 (TrBs) containing 50 µg/ml of protein.

After soaking for about 1 minute, the discs were blotted lightly, placed on filter paper (plain side up) and dried at 37°C for 30 minutes.

Antiserum was diluted in 1 per cent Tween 80/TrBs (Sigma Chemical Co. Ltd) and 0.2 ml volumes placed in the wells of plastic Disposo trays (Model 96 WS white; Linbro Chemical Co. Inc., New Haven, Conn., U.S.A.). The Tween 80 prevented non-specific adsorption of protein onto the discs. Dried antigen coated discs were placed in the wells with their plain sides in contact with the serum. The trays were rotated at 120 - 180 rpm for 45 minutes at room temperature on a Luckam rotator (Luckam Ltd.). After incubation in the antiserum the discs were given 5 by 5 minute washes in 1 ml of TrBs. They were rotated at 120 - 180 rpm between changes. Next 0.2 ml of FITC conjugate (Nordic Immunochemical Laboratories Ltd.) diluted in 2 per cent Tween 80/TrBs was placed over each disc. They were then incubated for a further period of 30 minutes at room temperature while being rotated at 120 - 180 rpm. The 5 by 5 minute washes in 1 ml of TrBs were then repeated. The washed discs were finally placed in the wells of a Titertex Linbro, Microtitre tray (Flow Laboratories Ltd.) and dried at 37°C for about 1 - 2 hours. The fluorescence on the discs were measured on a spectrofluorimeter against a saline coated control disc, using an excitation wavelength of 465 nm.

The controls for SAFA are summarised in Table 3. As a preliminary measure all of these were set up, but for practical purposes, since most of the controls gave negligible readings only those marked (*) were included routinely.

Table 3 Control reactions for SAFA.

Control name	Procedure		
	Antigen	Serum	Conjugate
Positive serum control / test*	+	+	+
Antigen control*	-	+	+
Conjugate control	+	+	-
Normal serum control*	+	+	+
Antigen control*	-	+	+
Conjugate control	+	+	-
Saline control*	+	-	+
Antigen saline control	-	-	+
Saline conjugate control	+	-	-

These were positive serum controls - using serum from known T. saginata infected cattle with either antigen or saline coated SAFA discs and an otherwise standard technique.

Normal serum controls - using serum from known uninfected cattle with either antigen or saline coated SAFA discs and

an otherwise standard technique.

Saline control - saline was added to the antigen coated SAFA disc with an otherwise standard technique.

As a standard procedure the reading for a serum coated SAFA disc was subtracted from the reading of the corresponding antigen and saline coated control disc. If the reading for a test SAFA disc was greater than that from a disc coated in normal serum, the test serum was considered to contain antibodies against a component of the T. saginata antigen.

2.7.12.1. Measuring the fluorescence of the SAFA discs.

A Pye Unicam SP860 (Pye Unicam Ltd.) fluorescent accessory was attached to a Pye Unicam SP1,800 spectrophotometer (Pye Unicam Ltd.) to measure the fluorescence on the SAFA discs. This apparatus compares logarithmically the ratio of the intensities of fluorescent emission in reference and sample solutions. Conventionally a solution of fluorescein at a dilution which is virtually free of self absorption effects but is sufficiently strong to be independent of small changes in concentration is placed in the reference cuvette. This solution then has a fluorescent emission of constant strength which can be compared to that of a sample solution.

Certain alterations had to be made to the SP 860 before the apparatus could be used to read the fluorescence on the SAFA discs. The makers suggest that the fluorescence of solid samples can be measured by mounting the sample at

45 degrees to the sample beam in the position normally occupied by the sample cell. However, as the fluorescence of the SAFA discs was very low relative to that of reference solution, an adequate differentiation between different positive SAFA discs was not obtainable by following the makers instructions.

An apparatus was constructed (Figure 11a) which would enable comparison of the fluorescence on a SAFA disc with that of a saline coated control disc. The relative difference between reference and sample beams was therefore maximised. The apparatus held a SAFA disc on one side and a saline coated piece of cellulose acetate filter on the other side as control. Figure 11b illustrates the optical arrangement of the apparatus. The sample disc is placed in the conventional reference beam since it is the sample disc which fluoresces brightly. The control disc is placed in the conventional sample beam since it does not fluoresce as brightly as the positive SAFA disc. The logarithmic ratio of the light intensities of the control and sample discs can then be compared.

The best filter combination for the spectrofluorimeter blocks out all the excitation wavelength reflected from the SAFA discs and only allows the fluorescent wavelength to pass through into the photomultiplier. The best excitation wavelength for use with FITC labelled antisera is 495 nm and the peak fluorescent transmission is at 525 nm. Several different filter combinations were tried and

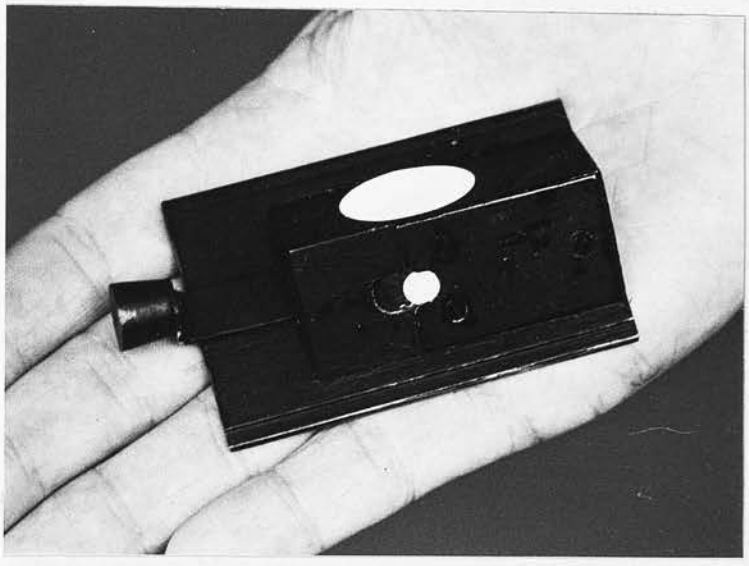


Figure 11a. The holder for the SAFA discs.

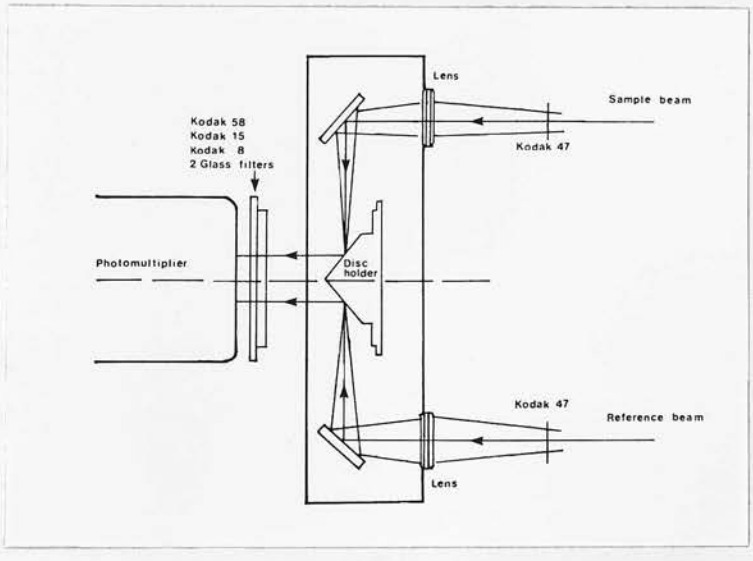


Figure 11b. The optical diagram for the SP 860 showing the positions of the SAFA disc holder and the filters.

the absorption spectra of the ones selected are shown in Figure 12.

Blue bandpass filters (Kodak Wratten Gelatin No.47, Kodak Ltd., London) which transmit maximally at 450 nm were placed in the light path before the sample and control discs. These filters cut off almost all the extraneous light coming through the monochromator. The filtered light then hit the control and sample discs.

Before entering the photomultiplier the light emitted or reflected from the discs passed through another set of Kodak gelatin filters Nos. 58, 15 and 8 (Kodak Ltd.) these were held together by two plain glass filters. Because of the low intensity of the light after passing through the filters the slit width was opened to 1 mm which corresponded to a bandwidth of 3 nm. An excitation wavelength of 465 nm was used as this gave maximal readings.

The filter combination was not optimal for two reasons; the excitation wavelength was only 465 nm and the secondary filters were cutting out some light at 525 nm, the peak of the emission spectrum for FITC. The suboptimal conditions were primarily due to the unexpectedly large amount of extraneous light which appeared to come from the monochromator of the spectrofluorimeter. This light had to be blocked out by filters as otherwise it reduced the reading obtained by lowering the proportionate difference between the light intensities of the two beams. The conditions used were the best obtainable with this particular apparatus and as can be seen from

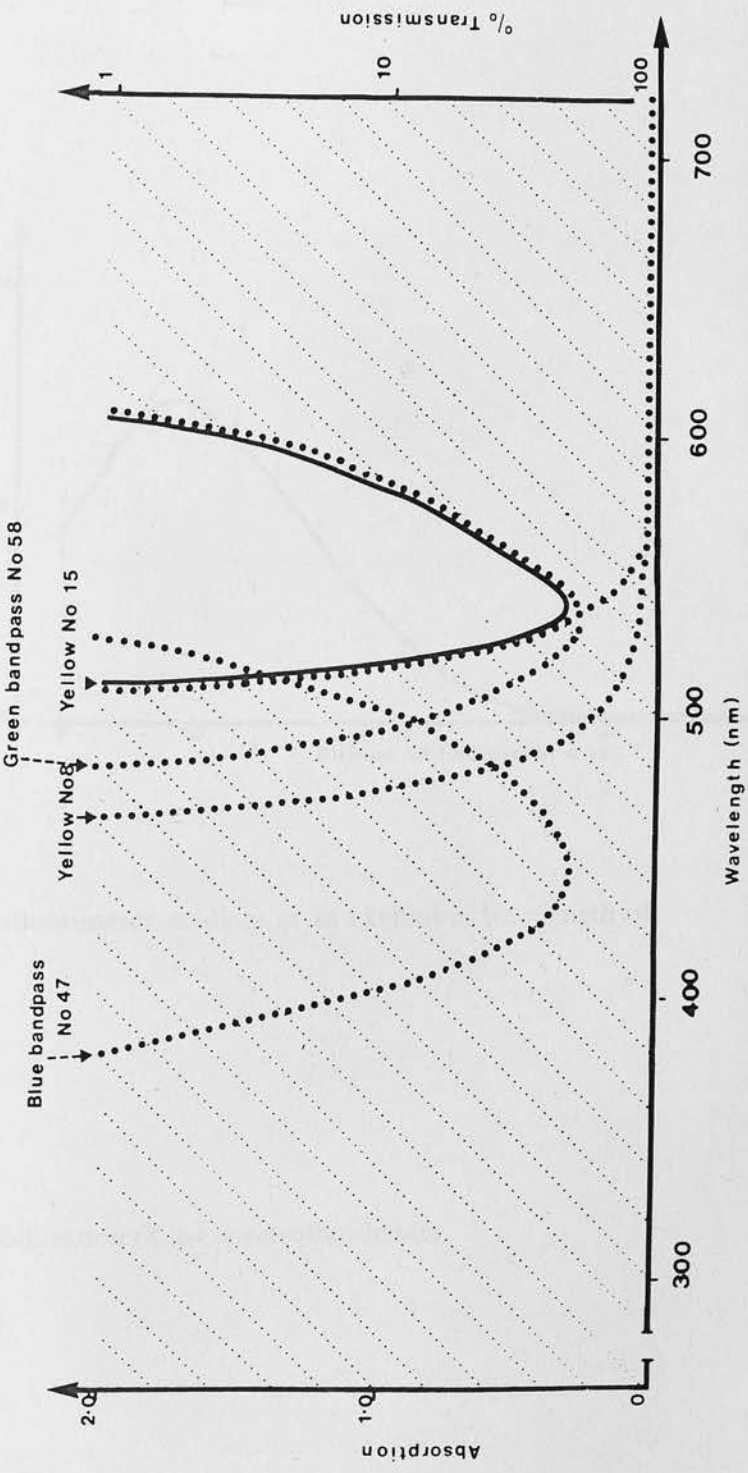
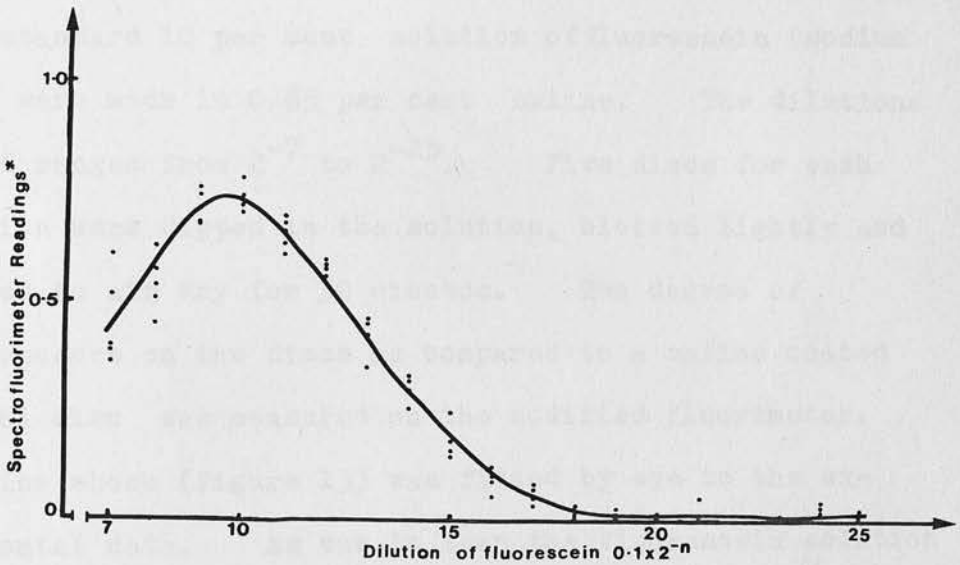


Figure 12. The absorption spectra of the filters used in the spectrofluorimeter for the SAFA technique.



* Spectrofluorimeter readings at an excitation wavelength of 465 nm.

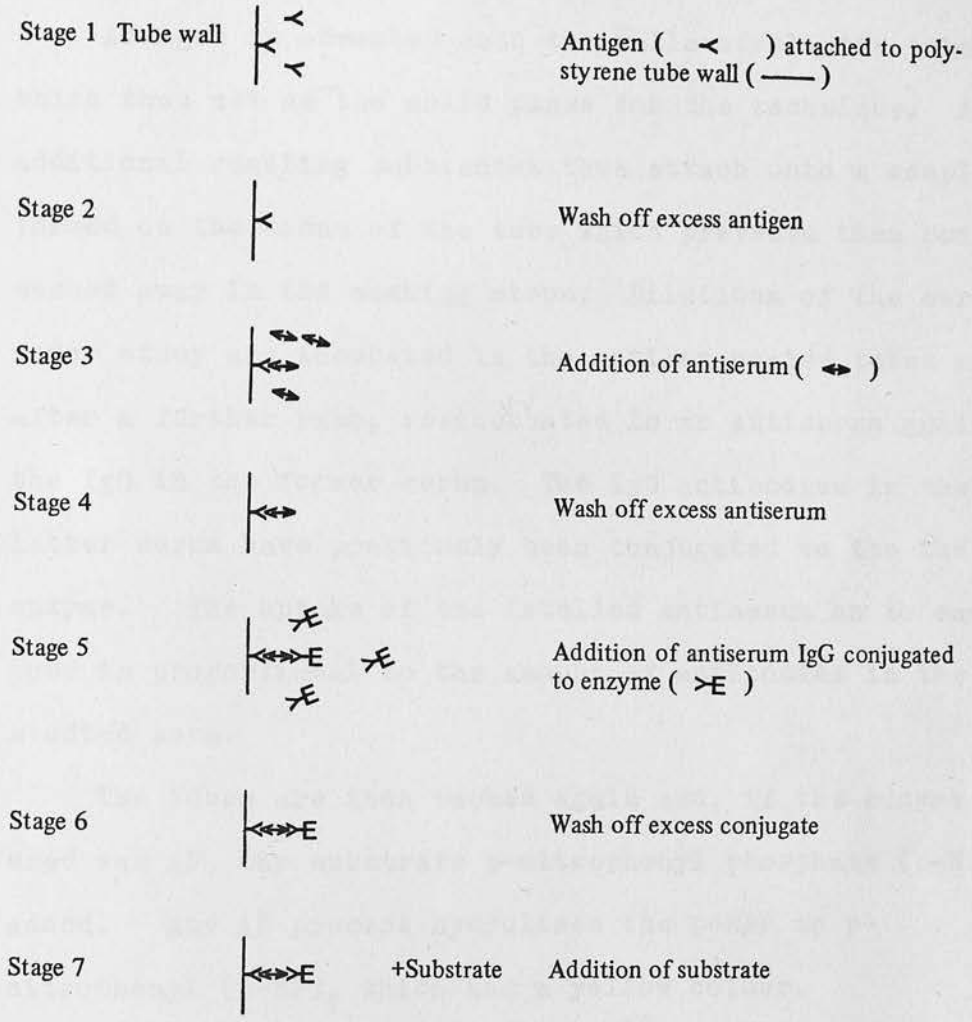
Figure 13. Calibration of the spectrofluorimeter.

the calibration graph (Figure 13) the apparatus was very sensitive.

The fluorimeter had to be calibrated in order to determine the relationship between fluorescein concentration and the readings obtained. Doubling dilutions of a standard 10 per cent solution of fluorescein (sodium salt) were made in 0.85 per cent saline. The dilutions tested ranged from 2^{-7} to 2^{-25}. Five discs for each dilution were dipped in the solution, blotted lightly and allowed to air dry for 30 minutes. The degree of fluorescence on the discs as compared to a saline coated control disc was measured on the modified fluorimeter. The line shown (Figure 13) was fitted by eye to the experimental data. As can be seen the fluorescein solution could still be detected at a dilution of 2^{-17}, which is equivalent to the discs being coated in a solution containing approximately 760 ng/ml of fluorescein, only a tiny proportion of which actually adheres to the cellulose acetate disc. The decrease in readings at high concentrations is due to self absorption by the fluorescein.

2.7.13. The enzyme-linked immunosorbent assay (ELISA).

The enzyme-linked immunosorbent assay (ELISA) for the quantitative assessment of serum antibody levels was first developed by Engvall and Perlmann (1972). ELISA (Figure 14) is essentially an indirect labelling technique using an antiserum conjugated to an enzyme such as alkaline phosphatase (AP). A colour change resulting from the



Stage 8 The degree of colour produced is measured on a spectrophotometer

Figure 14. A schematic representation of the stages in the enzyme-linked immunosorbent assay (ELISA).

enzyme's reaction with its substrate acts as the indicator system.

Antigen is adsorbed onto the walls of plastic tubes, which then act as the solid phase for the technique. All additional reacting substances thus attach onto a complex formed on the sides of the tube which prevents them being washed away in the washing steps. Dilutions of the sera under study are incubated in the antigen coated tubes and after a further wash, re-incubated in an antiserum against the IgG in the former serum. The IgG antibodies in the latter serum have previously been conjugated to the indicator enzyme. The uptake of the labelled antiserum on to each tube is proportional to the amount of antibodies in the studied sera.

The tubes are then washed again and, if the enzyme used was AP, the substrate p-nitrophenyl phosphate (p-NPP) added. Any AP present hydrolises the p-NPP to p-nitrophenyl (p-NP), which has a yellow colour.

p - nitrophenyl phosphate $\xrightarrow{AP}$ p-nitrophenyl + Pi

The degree of colour produced by the reaction after a standard time under standard conditions is measured on a spectrophotometer at 400 nm.

Shortly after Engvall & Perlmann (1972) introduced the ELISA technique, Ruitenberg, Steerenberg & Brozi (1975) reported a micro version of the technique which was carried out in flat bottomed Microtitre trays. These

authors used horse radish peroxidase (HRPO) as indicator enzyme instead of AP. The incubation times for the technique were reduced to 30 minutes at 37°C. Voller, Draper, Bidwell & Bartlett (1975) used micro- ELISA with AP as the indicator enzyme. In this work macro-ELISA using AP as indicator enzyme was used routinely.

2.7.13.1. Procedure for macro-ELISA - AP.

The technique (Figure 15) was essentially that of Engvall and Perlmann (1972) with slight modifications to the times of incubation in antiserum and conjugate. One ml of antigen containing 6 µg/ml protein in 0.1 M sodium carbonate buffer, pH 9.6, containing 0.02 per cent NaN_3 was added to 11 x 55 mm polystyrene tubes (Luckam Ltd.).

The capped tubes were incubated in a water bath at 37°C for 3 hours and stored overnight at 4°C for use the next day. The tubes were washed 3 times with 4 ml of 0.9 per cent NaCl containing 0.05 per cent Tween 20 (polyoxyethylene sorbiton monolaurate, Sigma Chemical Co. Ltd.) to prevent nonspecific adsorption onto the walls of the tubes. The washing solution was allowed to stand each time for 2 - 3 minutes before removal by suction pump.

One ml of bovine serum diluted in PBS pH 7.3 containing 0.02 per cent NaN_3 and 0.05 per cent Tween 20 (Sigma Chemical Co. Ltd.) was added to each tube. The tubes were incubated for 4 hours at room temperature and then given three successive 4 ml washes in the saline/Tween 20 solution. One ml of alkaline phosphatase labelled

Figure 15. Macro-ELISA tubes showing, from left to right, the colour reactions obtained with saline controls, normal serum controls and positive serum controls.

The substrate and saline control tubes should yield negligible extinction values. The small reading from the saline control (background reading) was substracted from the readings of the tubes incubated with serum. The difference between the ELISA readings using sera from cattle infected with T. saginata and uninfected cattle was then compared. If the ELISA reading from a serum sample was significantly greater than that for a normal serum control, then that serum was considered to contain antibodies to a component in the saline extract of T. saginata.

Table 4 Control reactions for ELISA.

Control Name	Procedure followed			
	Antigen	Serum	Conjugate	Substrate
Positive Control/ Test	+	+	+	+
Antigen control	-	+	+	+
Conjugate control	+	+	-	+
Normal Serum Control	+	+	+	+
Antigen control	-	+	+	+
Conjugate control	+	+	-	+
Saline control	+	-	+	+
Antigen saline control	-	-	+	+
Saline conjugate control	+	-	-	+
Antigen substrate control	+	-	-	+
Saline conjugate substrate control	+	-	-	-
Saline substrate control	-	-	-	+

2.7.13.2. Procedure for micro-ELISA.

The procedure for micro-ELISA (Figure 16) was basically the same as for macro-ELISA (2.7.13.1.). The main exceptions were that 0.3 ml volumes of reagents were used and the experiments were carried out in the wells of polystyrene Titertex/Linbro flat bottomed Microtitre trays (Flow Laboratories Ltd.). The washing procedure consisted of flooding the plates with 0.9 per cent saline solution containing 0.05 per cent Tween 20 (Sigma Chemical Co.Ltd.) and allowing them to soak for 5 minutes before emptying. The washing procedure was repeated 3 times.

In view of the small volume of reagent the absorption of the solution was read with 1 cm light path microcuvettes on a Pye Unicam SP 600 Spectrophotometer. The SP 1,800 Spectrophotometer could not be used with such small volumes because suitable cuvettes were not then available.

2.7.13.3. Horse radish peroxidase (HRPO) conjugate.

HRPO conjugate was used in the same way as AP conjugate. The dilution used was 1:100 and the incubation time in substrate was reduced to 60 minutes. The substrate for HRPO conjugate was 5 aminosalycylic acid (5AS; Cambrian Chemicals Ltd., Croydon) and H_2O_2.

The substrate was prepared as follows: 80 mg of 5AS was dissolved in 100 ml of hot distilled water. Directly prior to use the pH was adjusted 6.0 with 1 M NaOH. To 9 ml of the solution of 5AS, 1 ml of 0.05 per

Figure 16. Micro-ELISA plates showing the colour reactions obtained with positive serum controls (rows 1-4) normal serum controls (rows 6-9) and saline controls (rows 11 and 12).

cent H_2O_2 was added (Ruitenberg et al, 1975). The reaction of the enzyme and its substrate was stopped by the addition of a 0.025 ml drop of 4 M NaOH. The absorption of the solution was measured on a spectrophotometer at 449 nm.

2.7.13.4. The ELISA-inhibition technique.

This technique was used as a quick means of detecting antigen activity in purified extracts of T. saginata. The procedure for the technique involved mixing a volume of positive serum with an equal volume of the sample under test. The solutions were mixed and incubated for 30 minutes at 37°C before being diluted with PBS pH 7.3 containing 0.05 per cent Tween 20 (Sigma Chemical Co. Ltd.) to a dilution equivalent to the serum component being diluted to 1:50, the standard serum dilution for ELISA.

The ELISA technique was run according to standard procedure (2.7.13.1.). Additional controls (Table 5) were included for this technique along with those normally used for ELISA (Table 4). The ELISA technique was run as normal with a saline control and positive and normal serum controls. There should not be a statistical difference between the saline control values obtained in the ELISA-inhibition technique and the saline control values obtained for ELISA run in the normal way.

If for any test antigen sample, the absorbed serum reading was significantly less than the reading for the control serum dilution this was taken to indicate the presence of antigenic activity in the test sample.

Table 5 Additional controls for the ELISA-inhibition technique.

Control name	Procedure followed			
	Antigen	Positive serum	Normal serum	Saline
Positive serum/antigen control	+	+	-	-
Normal serum/antigen control	+	-	+	-
Saline/antigen control	+	-	-	-
Positive serum/saline control	-	+	-	+
Normal serum/saline control	-	-	+	+
Saline/saline control	-	-	-	+

CHAPTER 3

3. Results.

3.1. A study of the parameters of the SAFA technique.

3.1.1. Choice of material for the SAFA discs.

In certain fluorescent tests for bacteria, black cellulose filters were used in preference to white as the fluorescence on the bacteria showed up more clearly on the black background (Daniellson, 1965). An experiment was therefore designed to determine the more suitable colour of disc for use in SAFA. Dilutions of 10 per cent fluorescein in 0.85 per cent saline were made. Black and white Millipore HAWG cellulose acetate filter paper discs (Millipore (UK) Ltd.) were coated in various dilutions of fluorescein and allowed to dry, control discs were coated with normal saline. White discs were read against a white saline coated control disc and black discs against a black saline coated control disc.

Table 6 Spectrofluorimeter readings of black and white SAFA discs coated in various dilutions of fluorescein.

Dilution of fluorescein	Spectrofluorimeter readings at an excitation wavelength of 465 nm.	
	white discs	black discs
2,000	0.61	No reading
8,000	0.41	No reading
32,000	0.24	No reading
Saline	0.0	No reading

Unfortunately no readings were obtainable with the black discs (Table 6) at any of the fluorescein dilutions used. Attempts were made to clear the discs using Esso light parafin oil, but this procedure had no effect on the readings. Full scans using excitation wavelengths from 190 - 510 nm were also negative. White SAFA discs were therefore used throughout this work.

3.1.2. Method of drying the SAFA discs.

Two methods of air-drying SAFA discs were tested. Two groups of SAFA discs were dried at 37°C for 30 minutes and another 2 sets were dried overnight at room temperature. (There were 4 SAFA discs in the positive serum groups and 3 in the normal serum groups). The technique was carried out according to standard procedure. The results (Table 7) indicated that there was no significant difference ($p > 0.05$) between the two methods of drying the discs. Drying at 37°C for 30 minutes was, therefore, selected as this was more convenient. The full data for this experiment is given in appendix 1a.

Table 7 Comparison of method of drying SAFA discs.

Method of drying the SAFA discs.	Spectrofluorimeter reading * at an excitation wavelength of 465 nm.	
	Positive serum (E24)	Normal serum (N10)
	$\bar{x} \pm$ sd	$\bar{x} \pm$ sd.
30 minutes at 37°C	0.09 ± 0.01	0.02 ± 0.01
Overnight at room temperature	0.10 ± 0.01	0.02 ± 0.01

* Corrected to 2 decimal places (d.p.)

3.1.3. Fixatives for attaching antigen to the SAFA discs.

Various fixatives were used to attach the antigen to SAFA discs. After drying the antigen onto the discs in the usual manner they were either treated with 5 per cent formaldehyde, 95 per cent ethyl alcohol, 1 per cent acetic acid or left untreated. There were 4 SAFA discs in each positive serum group and 3 in each normal serum group. The three test groups were left soaking in the appropriate fixative for 10 minutes and then washed three times in TrBS. The SAFA technique was then carried out as usual. The results (Table 8) indicated that there was no significant difference between the readings obtained for positive serum with air dried or formalin fixed discs ($p > 0.05$), but that the readings for ethyl alcohol and acetic acid fixed discs were significantly less ($p < 0.01$). These results were similar to those found by Toussaint (1966). Simply air drying the discs was routinely used in the SAFA technique. The full data for this experiment is given in appendix 1b.

Table 8 Effect of various fixatives on the sensitivity of SAFA.

Fixative	Spectrofluorimeter readings* at an excitation wavelength of 465 nm.	
	Positive serum (E24) $\bar{x} \pm$ sd	Normal serum (N10) $\bar{x} \pm$ sd.
Air dried	0.15 ± 0.02	0.03 ± 0.01
5% formaldehyde	0.13 ± 0.03	0.02 ± 0.01
95% ethyl alcohol	0.08 ± 0.03	0.00 ± 0.01
1% acetic acid	0.10 ± 0.01	0.01 ± 0.00

* Corrected to 2 d.p.

3.1.4. Optimum time for incubation of the antigen/antibody complex.

The SAFA discs were treated in the normal manner except that three groups of 4 discs were incubated in antiserum for periods of 30, 45 and 60 minutes in order to determine whether the time of incubation of the antigen/antibody complex had any effect on the sensitivity of the SAFA technique (Table 9). There was no significant difference in the readings ($p > 0.05$). It is probable that the antigen/antibody complex forms very quickly, perhaps within seconds of the reagents being mixed. An incubation period of 45 minutes was selected for routine use in SAFA. The full data for this experiment is given in appendix 1c.

Table 9 Time of incubation of antigen/antibody complex.

Time of incubation of Ag/Ab complex	Spectrofluorimeter readings* at an excitation wavelength of 465 nm.	
	Positive serum (E24) $\bar{x} \pm$ sd.	Normal serum (N10) $\bar{x} \pm$ sd.
30 minutes	0.08 ± 0.02	0.02 ± 0.01
45 minutes	0.08 ± 0.01	0.00 ± 0.00
60 minutes	0.09 ± 0.01	0.02 ± 0.01

* Corrected to 2 d.p.

3.1.5. Antigen concentration.

Groups of 4 SAFA discs were coated in doubling dilutions of SE from 100 µg/ml to 6.75 µg/ml of protein. These groups of antigen coated discs were then incubated with positive serum diluted to either 1:2.5 or 1:5. Normal serum controls were also included. The results were expressed as the mean of the 4 SAFA readings. The normal serum began to show non-specific results at a protein concentration of 100 µg/ml (Figure 17). A dilution of 50 µg/ml antigen protein was selected as this gave negligible readings with normal serum and near maximal readings with the positive serum. One interesting point was that there was no significant difference ($p > 0.05$) in the readings for positive serum at a dilution of 1:5 for coating concentrations of 50 µg/ml and 100 µg/ml. antigen protein. This may have been because there was only a sufficient concentration of

Figure 17

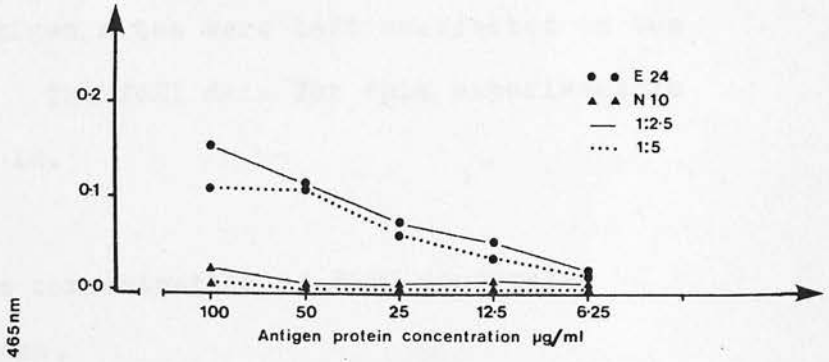


Figure 17. Antigen titration for the SAFA technique.

Figure 18

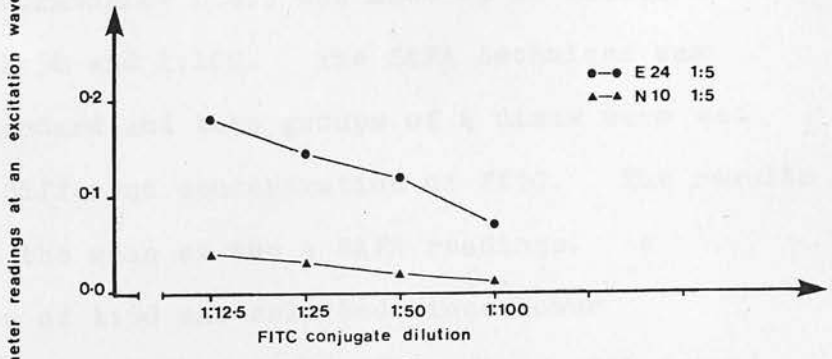


Figure 18. Conjugate titration for the SAFA technique.

Spectrofluorimeter readings at an excitation wavelength of 465nm

Figure 19

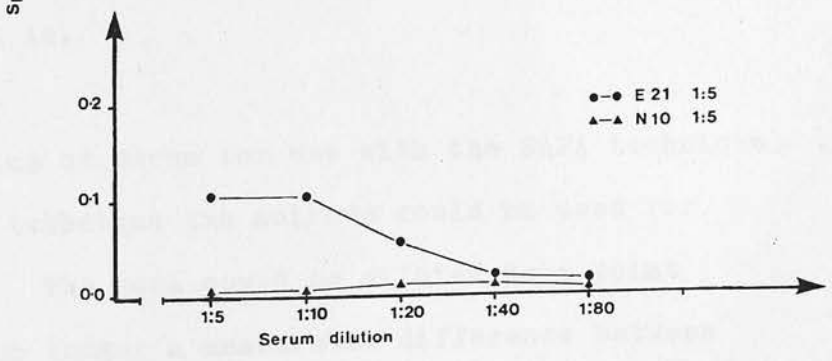


Figure 19. Serum titration for the SAFA technique.

antibody in the serum to fully coat the 50 µg/ml discs and therefore some antigen sites were left unaffected on the 100 µg/ml discs. The full data for this experiment is given in appendix 1d.

3.1.6. Optimum concentration of FITC conjugated antiserum.

Rabbit antibovine serum conjugated to FITC (Nordic Immunochemical Laboratories Ltd.) was made up in dilutions of 1:12.5, 1:25, 1:50 and 1:100. The SAFA technique was carried out as standard and then groups of 4 discs were each treated with a different concentration of FITC. The results were expressed in the mean of the 4 SAFA readings. A conjugate dilution of 1:50 was selected since lower dilutions gave appreciable non specific readings with normal serum (Figure 18). The full data for this experiment is given in appendix 1e.

3.1.7. Dilution of serum for use with the SAFA technique.

In the SAFA technique two methods could be used for comparing sera. The sera could be diluted to a point where there was no longer a measurable difference between the positive and normal sera and which was therefore taken as the end point or titre as with the IDH technique. An alternative procedure was to make one standard dilution for every serum and compare the degree of fluorescence with a standard positive serum, so obtaining a measure of the antibody level in the test serum.

Sets of 2 SAFA discs were first coated in antigen at a concentration of 50 µg/ml protein and then incubated with a serial dilution of either positive (E21) or normal serum (N10) from 1:5 - 1:80 (Figure 19). A dilution of 1:5 was selected as the most suitable for use in the SAFA technique as any higher concentration of serum would not have been feasible in terms of the amounts of reagents used. The full data for this experiment is given in appendix 1f. The SAFA and IDH titres for a group of 6 positive control sera were also compared (Table 10).

Table 10 A comparison of SAFA and IDH titres.

Sera	E19	E20	E21	E22	E23	E24
SAFA	1:5	1:5	1:20	1:20	1:20	1:40
IDH	1:16	1:8	1:128	1:32	1:64	1:512

The SAFA titres for the sera were estimated by making serial dilutions of the sera and testing each dilution in the SAFA technique. The last dilution of the serum with a SAFA reading higher than the normal serum control was considered the SAFA titre.

At this point it became obvious that SAFA was not as sensitive for measuring serum antibody levels as the IDH technique already in use. Since these high titre sera could at best only be diluted to 1:20 - 1:40 there was very little leeway for diluting sera, therefore low titre

sera were unlikely to be detected. The test would have to be performed at serum dilutions of about 1:5 and so would be costly in reagents.

3.1.8. The four layer SAFA technique.

Since the SAFA technique was not providing an acceptable degree of sensitivity the possibility of using a 'four layer' procedure was studied. The procedure was the same as with the indirect technique, with the following exceptions. After the 45 minute incubation period in the test serum the discs were washed in the normal manner. Next 0.2 ml of rabbit antibovine serum diluted to 1:5 in 1 per cent Tween 80 in TrBs was put on each disc. The discs were again incubated for 45 minutes, washed and 0.2 ml of FITC conjugated goat anti-rabbit serum (prepared by Mr. P.D. Le Riche) at a dilution of 1:25 in 1 per cent Tween 80 in TrBs was added. After a 30 minute incubation period the discs were washed and dried as usual and the degree of fluorescence was measured on the fluorimeter. The results, (Table 11) suggest that this technique gave significantly higher readings ($p<0.001$) for positive serum than the indirect technique. However, the technique was more involved, time consuming and more costly in terms of the amounts of reagents used than the conventional SAFA technique. The full data for this experiment is given in appendix 1g.

Table 11 Comparison of four layer technique with indirect SAFA technique

Test employed	Spectrofluorimeter readings* at an excitation wavelength of 465 nm.	
	Positive serum (E24) $\bar{x} \pm$ sd	Normal serum (N10) $\bar{x} \pm$ sd.
Four layer	0.24 ± 0.00	0.04 ± 0.02
Indirect	0.14 ± 0.01	0.02 ± 0.01

*Corrected to 2 d. p.

3.1.9. SAFA controls.

All the SAFA controls (Table 12) were set up using a standard serum dilution of 1:5. There were three SAFA discs in each group. As standard procedure the readings for a positive or normal serum control was substracted from the mean reading of the corresponding saline control. If the reading from the test disc was then still greater than that from a normal serum control, the serum was considered to contain antibodies against a component of the T. saginata antigen, i.e. the positive serum control minus the saline control = 0.09 and the normal serum control minus the saline control = 0.02 which gives a specific result of 0.01. The full data for this experiment is given in appendix 1h.

Table 12 SAFA controls.

Control name	Spectrofluorimeter readings* at an excitation wavelength of 465 nm.
Positive serum control/ test	0.11 ± 0.01
Antigen control	0.03 ± 0.00
Conjugate control	0.01 ± 0.01
Normal serum control	0.04 ± 0.01
Antigen control	0.00 ± 0.00
Conjugate control	0.00 ± 0.00
Saline control	0.02 ± 0.01
Antigen saline control	0.00 ± 0.00
Saline saline conjugate control	0.00 ± 0.00

*Corrected to 2 d.p.

3.2. A study of the parameters of the ELISA technique.

3.2.1. Stability of antigen coated ELISA tubes.

Twenty groups of 5 ELISA tubes were coated in 6 µg/ml of SE and then stored for periods of 1 - 4 weeks at 4°C prior to use in the ELISA technique. Each week 5 groups of tubes were used. They were incubated with either positive (E24) or normal serum (N10) at a dilution of 1:50, positive or normal serum at a dilution of 1:500 or in saline. Thereafter the regular procedure for macro-ELISA-AP was followed. The results (Figure 20), as with subsequent ELISA results, were obtained by subtracting the

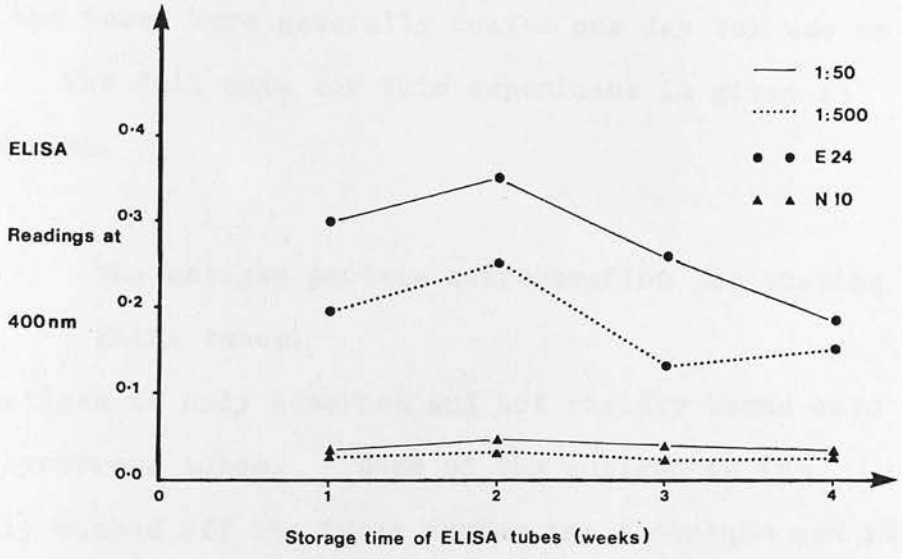


Figure 20. Stability of antigen coated ELISA tubes.

mean saline reading from each of the serum readings and calculating the mean and standard deviation for each group. The antigen coated tubes appeared to remain stable for up to 2 weeks after coating, but started to lose activity if stored longer than this. Since the coating process was so simple and the tubes could lose activity if stored for some time, the tubes were generally coated one day for use on the next. The full data for this experiment is given in appendix 2a.

3.2.2. The antigen protein concentration for coating ELISA tubes.

Antigen is only adsorbed and not rigidly bound onto the polystyrene tubes. Some of the antigen is inevitably washed off the tubes during the technique and it is therefore important that the tubes are coated with an optimum concentration of antigen. If the coating antigen solution is too concentrated too much antigen will be eluted from the tube. This free antigen will react with the antiserum in solution and, therefore, relatively less antiserum will be available to react with the antigen on the tube wall. This will result in low readings. With low antigen concentrations, not enough antigen will be available to react with the antiserum, again resulting in reduced readings.

Sets of 2 ELISA tubes were coated with concentrations of antigen from 60 µg/ml to 0.006 µg/ml of antigen protein and then incubated with either positive (E24) or normal (N10)

serum at dilutions of 1:50 and 1:500 (Figure 21).

A protein concentration of 6.0 µg/ml was selected as the concentration of antigen for coating the ELISA tubes. At higher concentrations the normal serum showed non-specific readings and there was little increase in the ELISA readings. In retrospect, a lower concentration of antigen (1.0 µg/ml) may have been a better choice. Linear rather than logarithmic dilutions of antigen might also have given a clearer indication of the best antigen concentrations. The full data for this experiment is given in appendix 2b.

3.2.3. Time of incubation in antiserum.

Groups of 2 ELISA tubes were coated in 6 µg/ml of antigen protein and then incubated in either positive (E24) or normal serum (N10) at dilutions of 1:50 or 1:500 for periods between 1.5 - 7.5 hours. The experiment then followed routine procedure.

The results (Figure 22) indicate that most of the antigen antibody reaction occurs quickly, certainly within 1.5 hours. The reading then increases slowly with time. The time of incubation in antiserum would not appear to be very critical in its effect on the sensitivity of the technique, any time between 1.5 and 7.5 hours would be satisfactory. The full data for this experiment is given in appendix 2c.

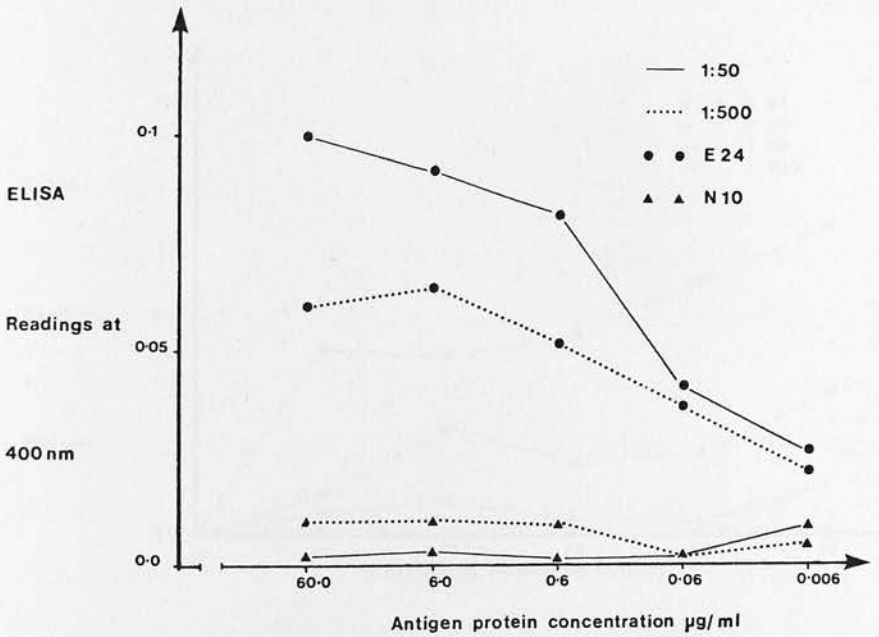


Figure 21. Antigen titration for the ELISA technique.

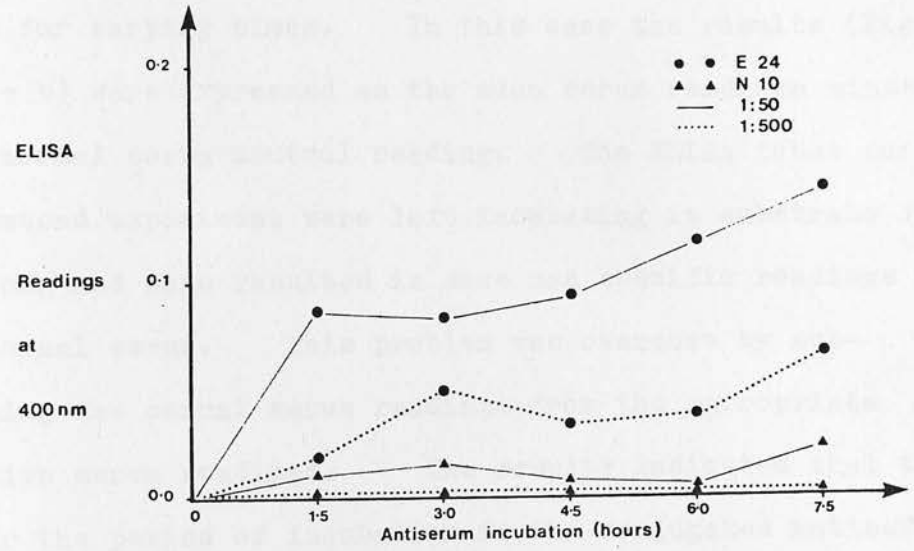


Figure 22. Kinetics of the antigen / antibody reaction for the ELISA technique.

3.2.4. Time of incubation in conjugate.

The time of incubation of the serum coated tubes in conjugated antiserum was varied between 1 and 24 hours. Sets of 2 tubes were treated according to the normal procedure, but were finally incubated in the conjugated antiserum for varying times. In this case the results (Figures 23 a + b) were expressed as the mean serum readings minus the mean normal serum control reading. The ELISA tubes for the second experiment were left incubating in substrate for too long and this resulted in some non specific readings in the normal serum. This problem was overcome by subtracting the normal serum readings from the appropriate positive serum readings. The results indicated that the longer the period of incubation in the conjugated antiserum, the more sensitive the technique. This result was similar to that of Engvall & Perlmann (1972). The relatively poor results at the shorter incubation times may be due to steric hindrance caused by the large enzyme molecules complexed to the globulin molecules. Therefore, for best results the conjugate incubation time at room temperature should be about 7 hours or preferably overnight. The full data for these experiments is given in appendix 2d and appendix 2e.

3.2.5. Fitting macro-ELISA - AP into a two day schedule.

The results from the previous two sections indicated

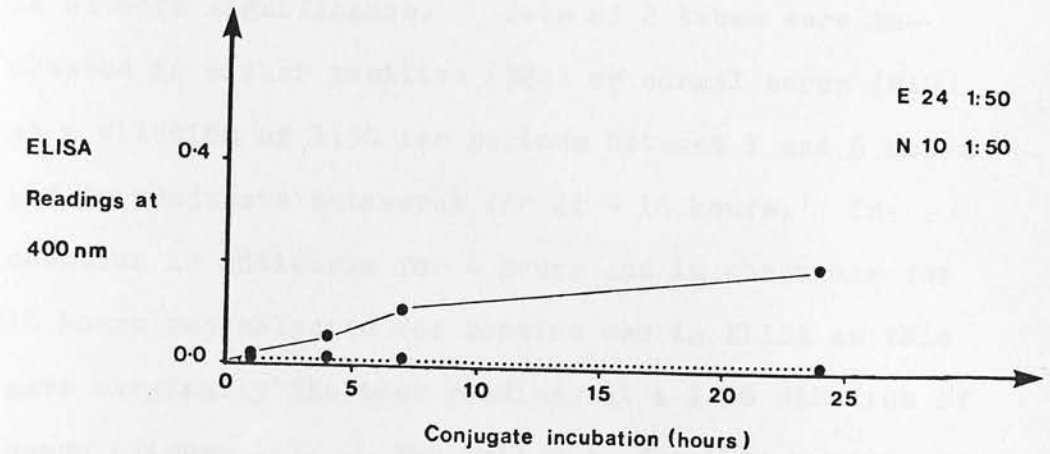


Figure 23a. Kinetics of the binding of the enzyme conjugate for the ELISA technique.

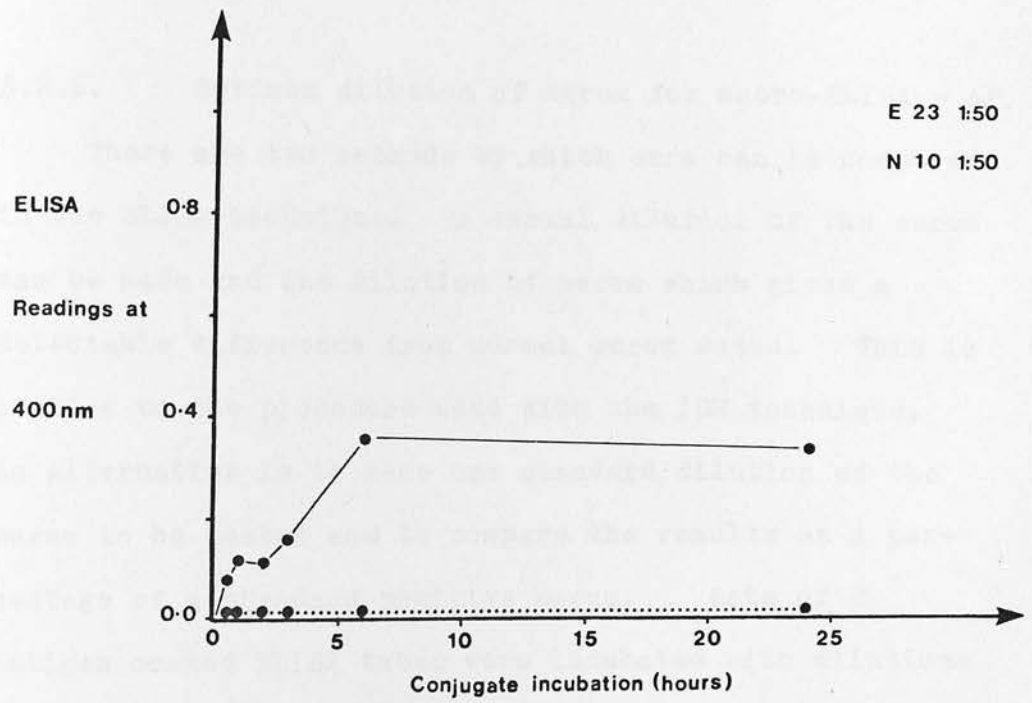


Figure 23b. Results of a repeat experiment on the kinetics of the binding of the enzyme conjugate for the ELISA technique.

that the time of incubation in the antiserum is not critical whereas the time of incubation in the conjugate is of more significance. Sets of 2 tubes were incubated in either positive (E24) or normal serum (N10) at a dilution of 1:50 for periods between 1 and 6 hours and in conjugate antiserum for 21 - 16 hours. Incubation in antiserum for 4 hours and in conjugate for 18 hours was selected for routine use in ELISA as this gave marginally the best readings at a 1:50 dilution of serum (Figure 24). The full data for this experiment is given in appendix 2f.

3.2.6. Optimum dilution of serum for macro-ELISA - AP.

There are two methods by which sera can be compared in the ELISA technique. A serial dilution of the serum can be made and the dilution of serum which gives a detectable difference from normal serum noted. This is similar to the procedure used with the IDH technique. An alternative is to make one standard dilution of the serum to be tested and to compare the results as a percentage of a standard positive serum. Sets of 2 antigen coated ELISA tubes were incubated with dilutions of either one of two positive sera (E24 and E21) and one normal serum (N10) at doubling dilutions from 1:5 to 1:10,240. The results (Figure 25) suggested that ELISA compared favourably with the IDH technique. Positive serum E24 had an IDH titre of 1:2,048, but in

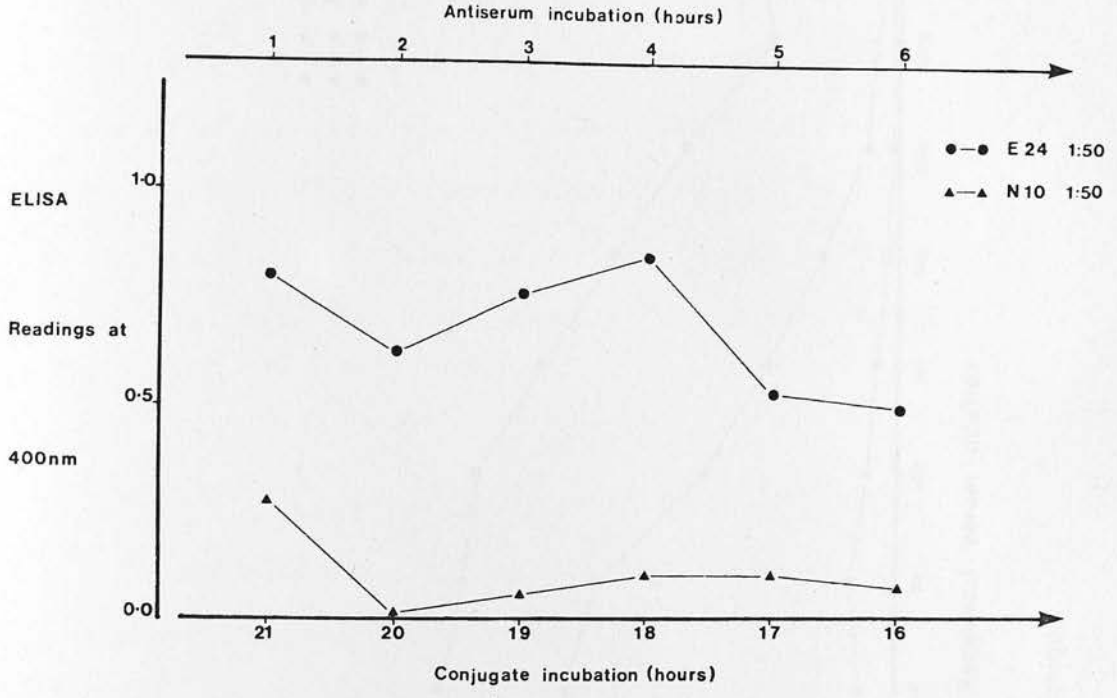


Figure 24. Fitting ELISA into a two day schedule.

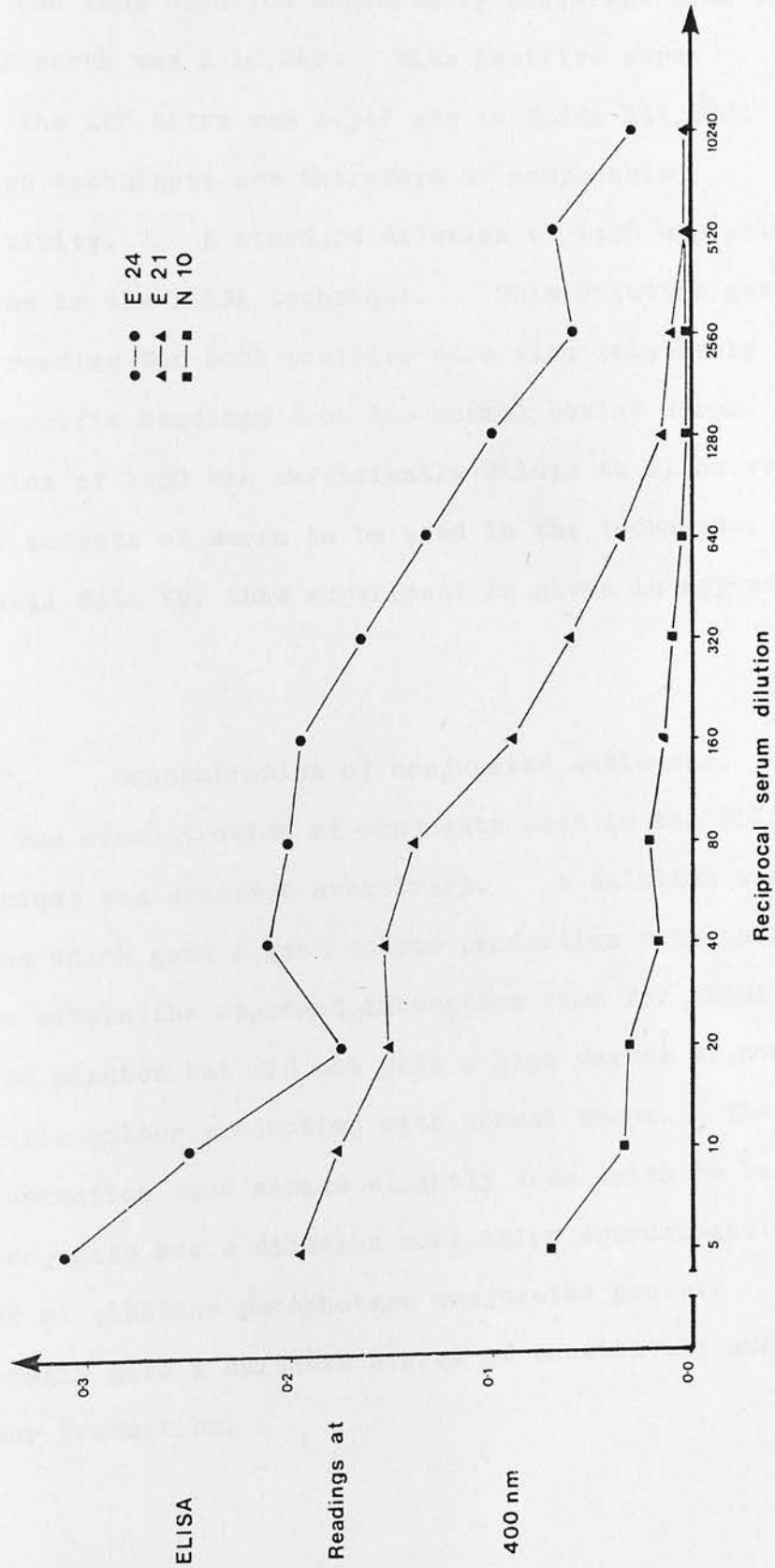


Figure 25. Serum titration for the ELISA technique.

ELISA the last dilution measureably different from the normal serum was 1:10,240. With positive serum E21 the IDH titre was 1:512 and in ELISA 1:1,280. The two techniques are therefore of comparable sensitivity. A standard dilution of 1:50 was selected for use in the ELISA technique. This dilution gave a high reading for both positive sera with relatively low non specific readings from the normal bovine serum. A dilution of 1:50 was sufficiently dilute to allow very small amounts of serum to be used in the technique. The full data for this experiment is given in appendix 2g.

3.2.7. Concentration of conjugated antiserum.

The concentration of conjugate used in the ELISA technique was somewhat arbritrary. A dilution was chosen which gave a good colour production with positive serum within the standard incubation time for ELISA - AP of 100 minutes but did not give a high degree of non-specific colour production with normal serum. The exact concentration used varied slightly from batch to batch of conjugate but a dilution containing approximately 1 µg/ml of alkaline phosphatase conjugated protein generally gave a suitable degree of sensitivity and colour production.

3.2.8. Incubation time of enzyme and substrate.

Figure 26 shows the colour production with time for an ELISA - AP test on a positive serum (E24). The indicator enzyme was AP which reacted with the substrate p-NPP. As can be seen the colour production is nearly linear up to an absorption of approximately 1.0 at 400 nm. This graph is essentially a reaction curve of the enzyme reaction at near optimal substrate concentration. That is, the substrate was in excess at least until an absorption of 1.0. The serum E24 was selected for this test because it was one of the highest titre sera available and the amount of enzyme conjugate on the tube was therefore as high as possible.

The enzyme reaction should therefore always be stopped before the solution has an absorption of 1.0. In practice ELISA - AP values, using a standard incubation time of 100 minutes were usually within 0.0 - 0.5 at 400 nm.

3.2.9. A comparison of the methods used to incubate ELISA tubes.

Engvall and Perlmann (1972) incubated their ELISA tubes on a roller drum whereas this procedure was omitted for micro-ELISA (Ruitenberg, Steerenberg & Brozi, 1975). Two sets of ELISA tubes were incubated for the standard times of 4 and 18 hours but one group

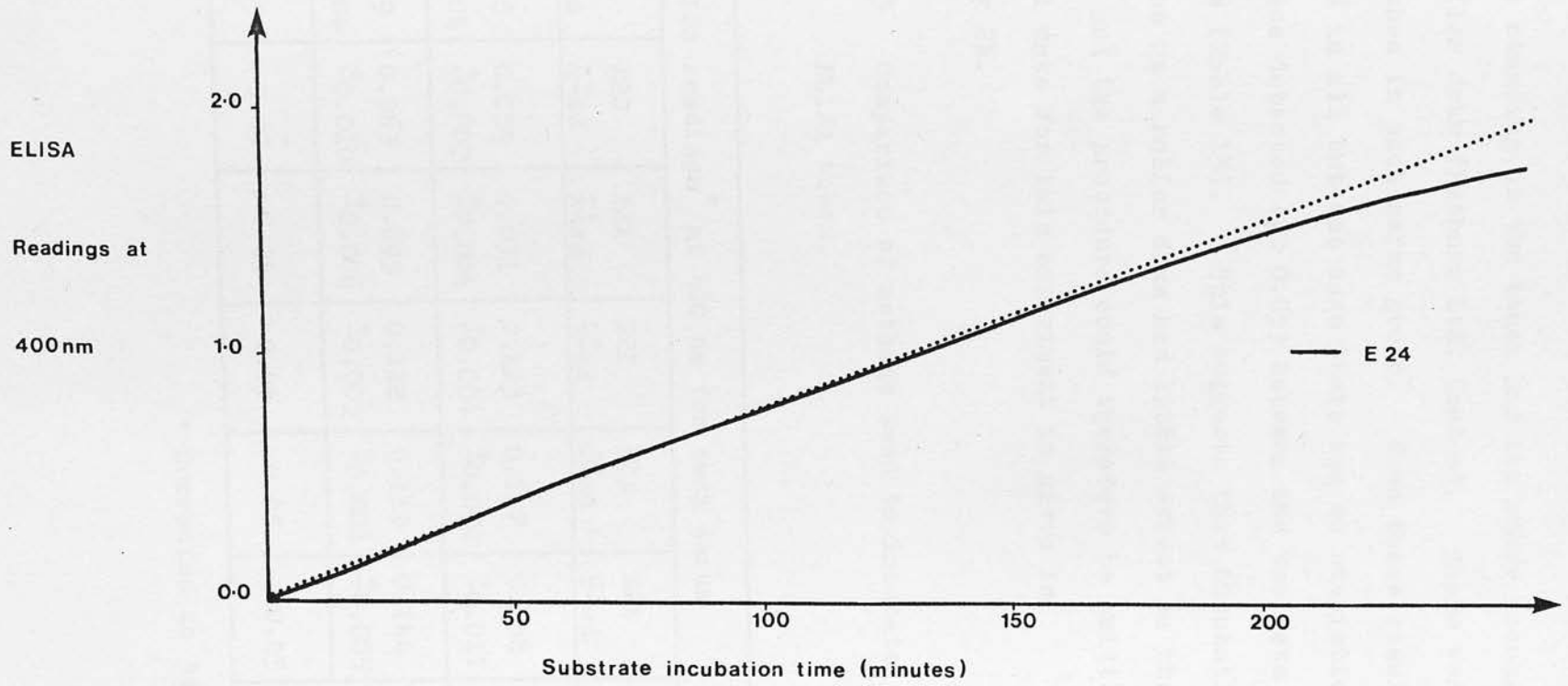


Figure 26. The colour reaction in the ELISA technique of the enzyme alkaline phosphatase with its substrate p-nitrophenyl phosphate with increasing time.

was left standing on the bench and the other incubated on a roller drum (Matburn Ltd. London). There were 5 ELISA tubes in each serum group. When these results were compared in all but one case there was no statistical difference detected ($p > 0.05$) between the two sets of readings (Table 13). This suggests that incubating the tubes on a roller drum had little effect on the results and the procedure could therefore be omitted. The full data for this experiment is given in appendix 2h.

Table 13 Comparison of methods used to incubate ELISA tubes.

Procedure	ELISA readings* at 400 nm for each serum							
	E19 $\bar{x} \pm sd$	E20 $\bar{x} \pm sd$	E21 $\bar{x} \pm sd$	E22 $\bar{x} \pm sd$	E23 $\bar{x} \pm sd$	E24 $\bar{x} \pm sd$	E25 $\bar{x} \pm sd$	N10 $\bar{x} \pm sd$
Roller drum	0.068 ±0.004	0.059 ±0.007	0.091 ±0.004	0.123 ±0.004	0.117 ±0.004	0.158 ±0.011	0.053 ±0.008	0.013 ±0.004
Standing still	0.069 ±0.004	0.063 ±0.010	0.093 ±0.006	0.122 ±0.005	0.119 ±0.008	0.144 ±0.005	0.056 ±0.004	0.009 ±0.006
Significance (p)	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05

* Corrected to 3d.p.

3.2.10. Calculation of the percentage standard deviation in macro-ELISA - AP.

Five groups of 20 tubes were incubated in 1:50 dilutions of one of three different positive sera, one normal serum or saline. The routine procedure for the ELISA technique was thereafter carried out. The results were expressed as a serum reading minus the mean saline control reading, the standard deviations were also included. The percentage standard deviation was calculated for each of the sera. The full data for this experiment is given in appendix 2i.

Table 14 The standard deviation within the ELISA technique.

Serum	ELISA readings* at 400 nm. ($\bar{x} \pm sd$)
E21	0.163 ± 0.030
E24	0.310 ± 0.054
E25	0.214 ± 0.041
N10	0.031 ± 0.053

* Corrected to 3 d.p.

The results indicated that the ELISA technique was quite consistent at least within one test. The percentage standard deviation was around about ± 18 per cent for

any one positive serum sample, although much greater for the normal serum controls.

3.2.11. Consistency of macro-ELISA - AP.

As the colour production in the ELISA technique was dependent on an enzyme/substrate reaction, the degree of colour produced by the same serum sample could vary slightly from day to day. Minor differences in temperature or in the time that the reaction was allowed to proceed could account for this. There were two possible solutions to this problem. A standard positive serum could have been set up with each experiment and the colour production of the enzyme substrate reaction monitored continuously on a spectrophotometer. The reaction in the other experimental tubes could have been stopped, when the standard reached a specified absorption reading. An alternative was to incubate all the tubes for a standard length of time. All the readings were then compared with a standard positive serum reading and the results expressed as a percentage of the standard. This was the technique used in this project. Figure 27a is an example of the variation that arose in the colour production of the enzyme reaction. Factors such as temperature, time of reactions, can result in this variation, but in this case it was probably due to different batches of enzyme conjugate being used. The regression lines AB and CD were calculated from week 4 to week 32 post infection for a calf given an oral infection with

100,000 T. saginata eggs at 3 months of age (E6). Analysis showed a statistical difference ($p < 0.05$) in the slope of the regression lines. The variance ratio between the regression coefficients was 6.7 (d.f. 1,24: $p < 0.05$). There was also a significant difference ($p < 0.001$) in the height of the two lines above the x-axis. The variance ratio between the regression constants was 239.4 (d.f. 1,24; $p < 0.001$).

If the results were expressed as a percentage of a standard positive serum (E24) these differences were no longer apparent (Figure 27b). The regression lines EF and GH were calculated from week 4 to week 32 post infection with the results expressed as a percentage of a standard positive serum (E24). Analysis showed no statistical difference ($p > 0.05$) in the slope of the regression lines. The variance ratio between the regression coefficients was 0.09 (d.f. 1,24: $p > 0.05$). Also there was no significant difference in the height of the two lines above the x - axis. The variance ratio between the regression constants was 0.43 (d.f. 1,24: $p > 0.05$). The full data for this experiment is given in appendix 6 and appendix 7.

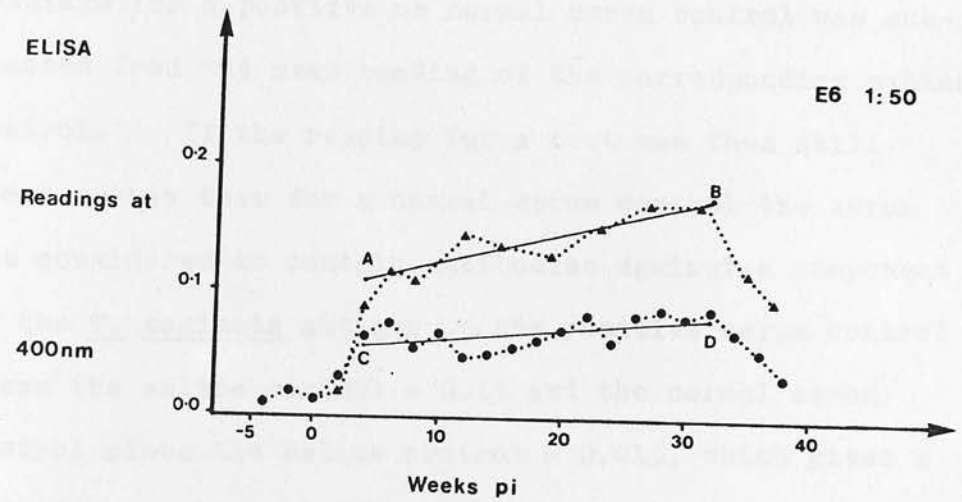


Figure 27a.

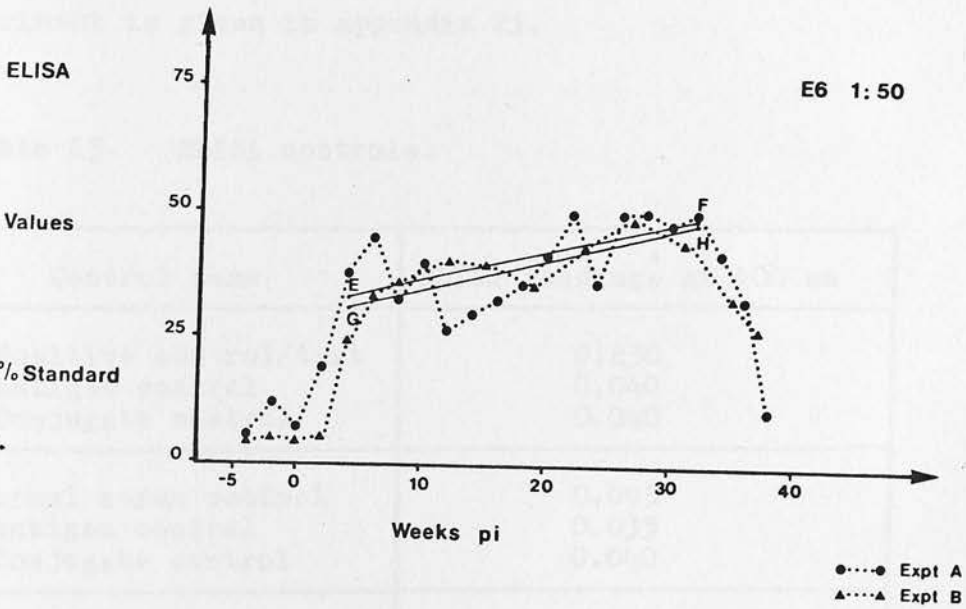


Figure 27b.

Figure 27. The difference in ELISA readings obtained on two separate occasions when monitoring the response of an experimental animal to oral infection with T. saginata eggs (Figure 27a) and how this discrepancy can be overcome by expressing the ELISA readings as a percentage of a standard positive serum (Figure 27b).

3.2.12. ELISA controls.

All the ELISA controls were set up using a standard serum dilution of 1:50. As standard procedure the readings for a positive or normal serum control was subtracted from the mean reading of the corresponding saline control. If the reading for a test was then still greater than that for a normal serum control the serum was considered to contain antibodies against a component of the T. saginata antigen i.e. the positive serum control minus the saline control = 0.15 and the normal serum control minus the saline control = 0.015, which gives a specific result of 0.135. The full data for this experiment is given in appendix 2j.

Table 15 ELISA controls.

Control name	ELISA readings* at 400 nm
Positive control/test Antigen control Conjugate control	0.230 0.040 0.040
Normal serum control Antigen control Conjugate control	0.095 0.035 0.040
Saline control Antigen saline control Saline conjugate control	0.080 0.035 0.040
Antigen substrate control Saline conjugate substrate control	0.035 0.040
Saline substrate control	0.045

*Corrected to 3 d.p.

3.2.13. A short comparison of micro and macro-ELISA for different incubation times and with either AP or HRPO as indicator enzyme.

Unfortunately since slightly different methods were used to read the results of micro and macro-ELISA (2.7.13 and 2.7.13.2.) most of the results could not be compared directly as ELISA readings (Tables 16a and 16c). For comparison, therefore, the results were expressed as a percentage of a standard positive serum (E24) (Tables 16b and 16d).

However, the times of incubation could be compared directly (Table 17a). In general the 4 plus 18 hour incubation period gave increased ELISA readings over the 30 plus 30 minutes at 37°C incubation, but there were some instances (*) where there was no significant difference. This discrepancy was probably related to the results obtained in varying the times of incubation in conjugate (3.2.4.), when shorter incubation periods in conjugate resulted in lower ELISA readings. When these results were expressed as a percentage of a standard positive serum, the difference largely disappeared (Table 17b), although there were some exceptions (*).

As was to be expected there was a difference in the readings obtained in ELISA for the two indicator enzymes, because of the different colour production of these enzymes. However, when the readings were compared, expressed as a percentage of a standard positive serum (E24), (Table 17c) this difference was reduced considerably, but some of the

results (*) were still significantly different.

A significant difference was not detected between micro and macro-ELISA when the results were expressed as a percentage of a standard positive serum (E24), at least for HRPO. With ELISA-AP, however, although the results were similar most of the ELISA values were statistically different (Table 17d). The full data for these experiments is given in appendix 2k. In tables 16a - d, incubation time A was 4 hours in antiserum and 18 hours in conjugate at room temperature. Incubation time B was 30 minutes in antiserum and 30 minutes in conjugate at $37^{o}C$.

Table 16a Micro - ELISA using different incubation times and either AP or HRPO as the indicator enzyme. The results are expressed as ELISA readings.**

	Procedure and procedure coding	Serum							
		E19 $\bar{x} \pm$ sd	E20 $\bar{x} \pm$ sd	E21 $\bar{x} \pm$ sd	E22 $\bar{x} \pm$ sd	E23 $\bar{x} \pm$ sd	E24 $\bar{x} \pm$ sd	E25 $\bar{x} \pm$ sd	N10 $\bar{x} \pm$ sd
A	Micro-ELISA-AP Incubation time A.	0.350* ±0.022	0.326 ±0.047	0.468 ±0.018	0.590 ±0.016	0.548 ±0.027	0.732 ±0.008	0.318 ±0.013	0.138 ±0.031
B	Micro-ELISA-AP Incubation time B.	0.224 ±0.010	0.242 ±0.008	0.338* ±0.005	0.410 ±0.007	0.402 ±0.013	0.468 ±0.040	0.216 ±0.010	0.088 ±0.015
C	Micro-ELISA-HRPO Incubation time A.	0.685* ±0.097	0.504 ±0.051	0.752 ±0.140	1.04 ±0.065	1.024 ±0.077	1.324 ±0.147	0.458 ±0.038	0.136 ±0.063
D	Micro-ELISA-HRPO Incubation time B	0.462 ±0.055	0.360 ±0.031	0.558 ±0.08	0.712 ±0.089	0.748 ±0.099	1.010* ±0.120	0.350 ±0.069	0.009 ±0.053

* Sample size= 4 instead of 5
** Corrected to 3 d.p.

Table 16b Micro - ELISA using different incubation times and either AP or HRPO as the indicator enzyme. The results are expressed as a percentage of a standard positive serum (E24).**

Procedure and procedure coding		Serum							
		E19 $\bar{x} \pm$ sd	E20 $\bar{x} \pm$ sd	E21 $\bar{x} \pm$ sd	E22 $\bar{x} \pm$ sd	E23 $\bar{x} \pm$ sd	E24 $\bar{x} \pm$ sd	E25 $\bar{x} \pm$ sd	N10 $\bar{x} \pm$ sd
A'	Micro-ELISA-AP Incubation time A	47.95* ± 2.98	44.58 ± 6.37	64.10 ± 2.47	80.82 ± 2.18	75.08 ± 3.67	100.20 ± 1.12	43.50 ± 1.79	18.9 ± 4.25
B'	Micro-ELISA-AP Incubation time B.	47.64 ± 1.88	51.50 ± 1.79	77.78* ± 1.05	87.22 ± 1.52	85.52 ± 2.74	99.58 ± 8.44	45.96 ± 1.88	18.74 ± 3.16
C'	Micro-ELISA - HRPO Incubation time A	52.70* ± 7.45	38.76 ± 3.95	57.84 ±10.80	79.90 ± 4.89	78.78 ± 5.90	101.80 ± 11.34	35.20 ± 2.90	10.44 ± 4.80
D'	Micro-ELISA - HRPO Incubation time B	45.74 ± 5.49	35.64 ± 3.03	55.24 ± 7.89	70.50 ± 8.80	74.06 ± 9.77	100.00 ± 11.90	34.66 ± 6.80	8.94 ± 5.24

* Sample size = 4 instead of 5
** Corrected to 2 d.p.

Table 16c Macro - ELISA using different incubation times and either AP or HRPO as the indicator enzyme. The results are expressed as ELISA readings.**

	Procedure and procedure coding	Serum							
		E19 $\bar{x} \pm$ sd	E20 $\bar{x} \pm$ sd	E21 $\bar{x} \pm$ sd	E22 $\bar{x} \pm$ sd	E23 $\bar{x} \pm$ sd	E24 $\bar{x} \pm$ sd	E25 $\bar{x} \pm$ sd	N10 $\bar{x} \pm$ sd
E	Macro-ELISA - AP Incubation time A	0.075 ±0.011	0.051 ±0.007	0.101 ±0.008	0.122 ±0.012	0.115 ±0.006	0.171 0.029	0.052 ±0.009	0.017 ±0.020
F	Macro-ELISA - AP Incubation time B	0.058 ±0.012	0.044 ±0.004	0.077 0.006	0.091 0.007	0.088 ±0.005	0.123 ±0.019	0.032 ±0.012	0.001 ±0.002
G	Macro-ELISA - HRPO Incubation time A	0.195 ±0.010	0.162 ±0.008	0.222 ±0.013	0.359 ±0.013	0.344 ±0.018	0.464 ±0.010	0.158 ±0.008	0.070 ±0.027
H	Macro-ELISA - HRPO Incubation time B	0.163 ±0.005	0.135 ±0.010	0.183 ±0.007	0.277 0.012	0.275 ±0.009	0.368 ±0.011	0.128 ±0.008	0.038 ±0.015

** Corrected to 3 d.p.

Table 16d Macro ELISA using different incubation times and either AP or HRPO as the indicator enzyme. The results are expressed as a percentage of a positive standard serum (E24).*

	Procedure and procedure coding	Serum							
		E19 $\bar{x} \pm$ sd	E20 $\bar{x} \pm$ sd	E21 $\bar{x} \pm$ sd	E22 $\bar{x} \pm$ sd	E23 $\bar{x} \pm$ sd	E24 $\bar{x} \pm$ sd	E25 $\bar{x} \pm$ sd	N10 $\bar{x} \pm$ sd
E'	Macro-ELISA - AP Incubation time A	44.12 ± 6.58	30.00 ± 4.38	59.40 ± 4.38	71.76 ± 6.79	67.64 ± 3.59	100.58 ± 16.8	30.00 ± 4.38	10.00 ±12.5
F'	Macro-ELISA - AP Incubation time B	48.32 ± 9.60	33.66 ± 3.50	64.16 ± 4.75	75.82 ± 5.40	74.27 ± 4.08	102.44 ± 16.02	28.30 ± 9.05	0.83 ± 1.86
G'	Macro-ELISA - HRPO Incubation time A	42.30 ± 2.20	35.24 ± 1.84	48.70 ± 3.30	77.38 ± 2.90	74.82 ± 3.92	100.88 ± 1.97	34.36 ± 1.84	15.20 ± 5.75
H'	Macro-ELISA - HRPO Incubation time B.	43.76 ± 0.77	36.46 ± 2.70	49.46 ± 1.85	74.88 ± 3.24	74.30 ± 2.54	99.46 ± 2.95	34.56 ± 2.26	10.18 ± 4.13

* Corrected to 2 d.p.

Table 17a Comparison of the ELISA readings obtained with differing incubation times - results expressed as ELISA readings.

Serum		E19	E20	E21	E22	E23	E24	E25	N10
Significance (p)	A x B	<0.001	< 0.01	<0.001	<0.001	<0.001	< 0.001	< 0.001	<0.001
	C x D	<0.01	< 0.001	< 0.05	< 0.001	< 0.01	< 0.01	< 0.02	<0.01
	E x F	<0.05	>0.05*	< 0.001	< 0.01	< 0.001	< 0.02	>0.05*	>0.05*
	G x H	<0.001	< 0.01	< 0.001	< 0.001	<0.001	< 0.001	<0.001	<0.05

Table 17b Comparison of the ELISA values obtained with different incubation times - results expressed as ELISA values.

Serum		E19	E20	E21	E22	E23	E24	E25	N10
Significance (p)	A' x B'	>0.05	< 0.05*	< 0.001*	<0.001*	<0.01*	>0.05	>0.05	>0.05
	C' x D'	>0.05	>0.05	>0.05	>0.05	>0.05	> 0.05	>0.05	> 0.05
	E' x F'	>0.05	<0.05*	>0.05	>0.05	<0.05*	>0.05	>0.05	>0.05
	G' x H'	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	> 0.05	>0.05

Table 17c Comparison of the ELISA values obtained using ELISA - AP and HRPO - results expressed as a percentage of a standard positive serum (E24).

Serum		E19	E20	E21	E22	E23	E24	E25	N10
Significance (p)	A' x C'	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	<0.001*	<0.01*
	E' x G'	>0.05	<0.05*	<0.01*	>0.05	<0.02*	>0.05	>0.05	>0.05
	B' x D'	>0.05	<0.005*	<0.001*	<0.01*	<0.05*	>0.05	<0.01*	<0.01*
	F' x H'	>0.05	>0.05	<0.001*	>0.05	>0.05	>0.05	>0.05	<0.01*

Table 17d Comparison of micro and macro ELISA results expressed as a percentage of a standard positive serum (E24).

Serum		E19	E20	E21	E22	E23	E24	E25	N10
Significance (p)	A' x E'	>0.05	<0.01*	>0.05	<0.05*	<0.02*	>0.05	<0.001*	>0.05
	B' x F'	>0.05	<0.001*	<0.001*	<0.01*	<0.001*	>0.05	<0.01*	<0.001*
	C' x G'	<0.02*	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	D' x H'	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

3.3. Immunochemistry.

3.3.1. Preliminary work.

3.3.1.1. Heat treatment.

Heating SE did not markedly affect the HI titre (Table 18), which was reduced by about two doubling dilutions after SE was treated at 100°C. Gel precipitin antigens 3 and 4 were lost on heating at 56°C but gel precipitin antigen 1 was still present following treatment at 65°C and gel precipitin antigen 2 was stable at 100°C for 30 minutes. Neither the protein nor carbohydrate content was affected greatly by heating although there was a slight reduction in both.

3.3.1.2. Trichloroacetic acid (TCA) precipitation.

Some protein and carbohydrate remained in the supernatant when an aliquot of SE was precipitated with 0.25M TCA (Table 18) even though this concentration of acid is normally sufficient to ensure complete precipitation of protein (Dawson et al, 1969). Most of the protein, carbohydrate and material showing HI activity was precipitated. Gel precipitin antigen 2 was also found in the precipitate but antigens 1, 3 and 4 could not be detected.

3.3.1.3. Ammonium sulphate precipitation.

A small amount of protein and carbohydrate was precipitated with 20 per cent ammonium sulphate (Table 18). Gel precipitin antigen 2 was detected in the precipitate but most of the antigenic activity occurred in the supernatant. More protein and carbohydrate were precipitated with 40 per cent ammonium sulphate as was most of the HI and gel precipitin activity, but there was still some antigen activity left in the supernatant. At 60 per cent or more ammonium sulphate nearly all the HI and gel precipitin activity along with most of the protein and carbohydrate was precipitated. As with TCA precipitation, gel precipitin antigens 1, 3 and 4 were lost.

Table 18 Preliminary work on SE.

Treatment		HI titre $\frac{1}{\text{Log } 2}$	Precipitins	Protein µg / ml	Carbo-hydrate µg / ml
Untreated SE		11	1,2,3, 4	2070	1567
Heat treatment					
56°C		11	1, 2	1955	1340
65°C		10	1, 2	1755	1330
76°C		10	2	1755	1330
100°C		9	2	1960	1470
Trichloro-acetic acid					
PPt		9	2	770	1110
SN		3	-	230	500
Ammonium Sulphate					
20%	PPt.	-	2	90	100
	SN	11	2	1710	1310
40%	PPt.	9	2	1260	980
	SN	6	2	680	590
60%	PPt.	10	2	1045	835
	SN	2	-	45	170
80%	PPt.	7	2	1000	800
	SN	-	-	70	80
100%	PPt.	9	2	1080	860
	SN	3	-	35	50

PPt. - precipitate

SN - supernatant

3.3.2. Chromatography of SE.

3.3.2.1. Analysis of fractions.

Unless otherwise stated the antigenic activity of sub-fractions of SE was tested on two separate occasions by the haemagglutination inhibition (HI) technique (2.7.6.). The HI titre of the fraction pools was checked again following their reconcentration by dialysis in visking tubing against carbowax (2.6.1.7.). Gel precipitin antigens in the concentrated fraction pools were detected by the micro gel precipitation technique usually by testing against serum E21 (2.7.7.). Protein estimations were carried out by the Folin technique (2.7.1.) and carbohydrate estimations by the anthrone reaction (2.7.4.).

3.3.2.2. Gel filtration.

3.3.2.2.1. Sephadex G200.

3.3.2.2.1.1. Molecular weight distribution in SE.

Samples of SE and of normal bovine serum were fractionated by Sephadex G200 gel filtration (Figure 28). The first peak from the saline extract was eluted in approximately the same position as the macroglobulin component of the bovine serum. This corresponded to molecules of a molecular weight of approximately 10^6 if a globular or 10^5 if a linear configuration. The second saline extract peak was rather diffuse but was eluted slightly

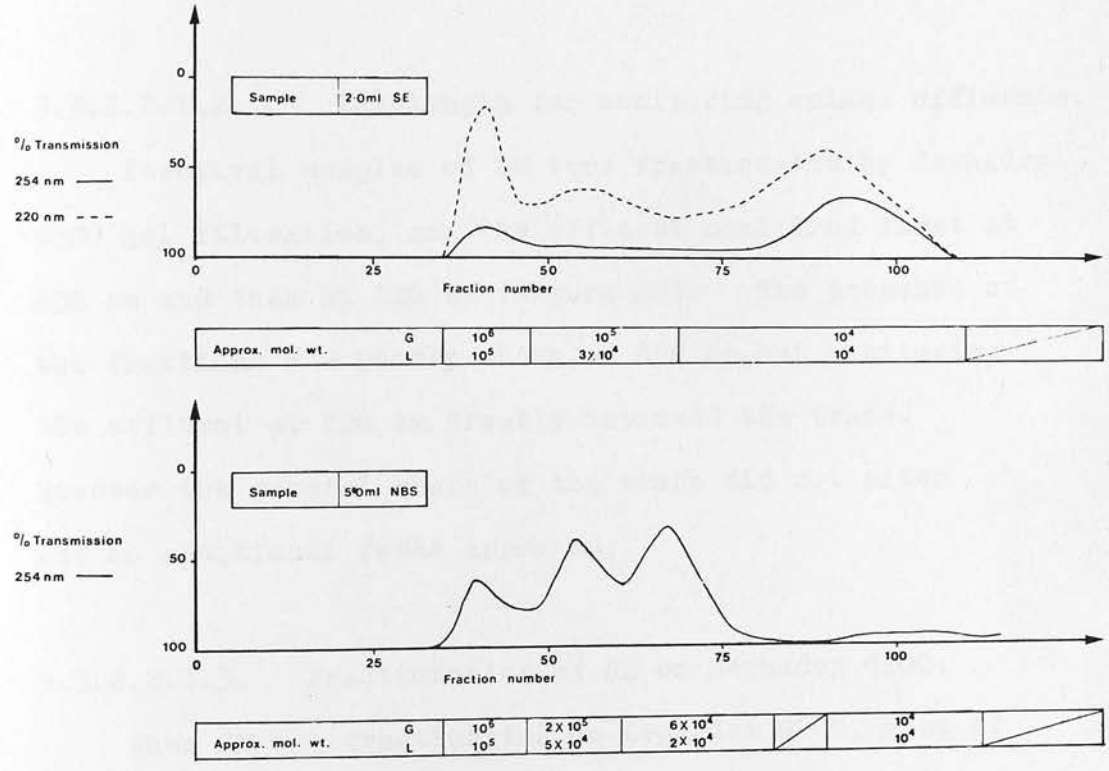


Figure 28. The elution patterns obtained by Sephadex G200 chromatography of SE and of normal bovine serum (NBS) illustrating the molecular weight distribution of the molecules in these samples.

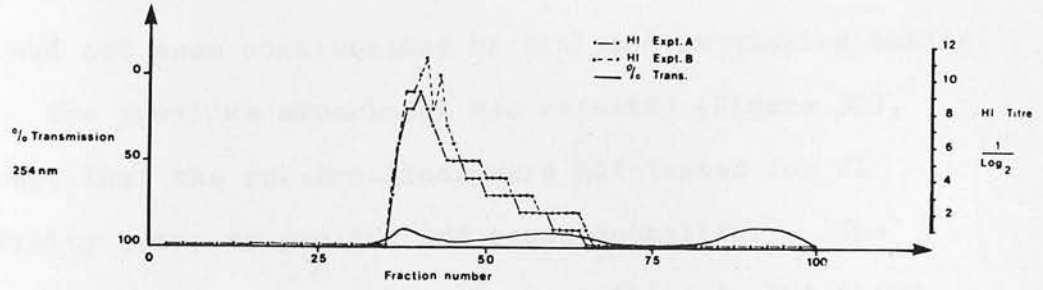
behind the 7s globulin peak and corresponded to molecules of a molecular weight of approximately 10^5 if globular or 3×10^4 if linear. The third saline extract peak and the fourth serum peak corresponded to small molecules.

3.3.2.2.1.2. Wavelength for monitoring column effluents.

Identical samples of SE were fractionated by Sephadex G200 gel filtration, and the effluent monitored first at 254 nm and then at 220 nm (Figure 28). The presence of the fractions was poorly shown at 254 nm, but monitoring the effluent at 220 nm greatly improved the trace. However the general shape of the trace did not alter and no additional peaks appeared.

3.3.2.2.1.3. Fractionation of SE on Sephadex G200.

When SE was fractionated on Sephadex G200, most of the HI activity was associated with the first peak from the column (Figure 29). The HI activity tended to spread into other fraction pools, but fraction pool 1.S.1. gave the highest HI titres. Gel precipitin antigen 1 was present in this fraction. Fraction pool 1.S.2. also contained HI activity but in addition it had gel precipitin antigens 2, 3 and 4. The third fraction pool 1.S.3. contained a very low concentration of HI activity, but no detectable gel precipitin antigens and fraction pool 1.S.4 did not contain any detectable antigenic activity.



Fraction pool		1.S.1.	1.S.2.	1.S.3.	1.S.4.	% Recovery
Pooled fractions		34–45	46–71	72–85	86–100	
Protein content µg		500 (23·1%)	770 (35·6%)	390 (18·0%)	150 (6·9%)	83·6%
Carbohydrate content µg		435 (11·8%)	565 (15·4%)	360 (9·8%)	190 (5·2%)	42·3%
HI Titres $\frac{1}{\text{Log}_2}$		7	6	< 2	–	
Gel precipitins		1	2 (3,4)	–	–	
Approx. mol. wt.	G	10^6	8×10^4	2×10^4	10^4	
	L	10^5	2×10^4	$1{\cdot}5 \times 10^4$	10^4	

Sample	2·0 ml SE
Protein content	2160 µg
Carbohydrate cont.	3660 µg
Flow rate	3·4 ml/ cm^2/ hr.
Fraction collection	4/hr.

Figure 29. Sephadex G200 chromatography of SE.

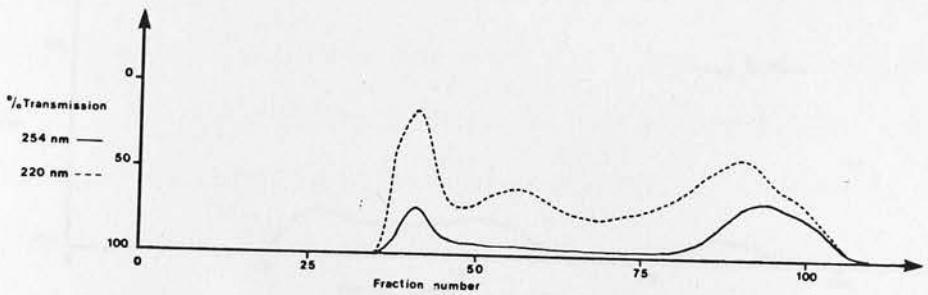
The majority of the protein applied to the column was recovered but very little carbohydrate was recovered. The ratio of carbohydrate to protein applied to the column was approximately 3:2 but the ratio recovered was only about 1:2. Before application to the column this sample of SE had not been concentrated or dialysed in visking tubing.

The previous experiment was repeated (Figure 30), except that the sub-fractions were not tested for HI activity prior to pooling and reconcentration. The results were similar to the first experiment, but there were some exceptions. There was a small amount of gel precipitin antigen 2 present in fraction pool 2.S.1. and gel precipitin antigen 1 was present at low concentration in fraction pool 2.S.2. Fraction pool 2.S.3. corresponded to fraction pools 1.S.3. and 1.S.4. of the first experiment. This fraction pool contained a small amount of HI activity and gel precipitin antigen 2 at a low concentration.

The sample of SE applied to the column had been re-concentrated by ultra-filtration using a membrane with a molecular weight cut off of 1,000. The ratio of carbo-hydrate to protein in this extract was very similar to that recovered from the column.

3.3.2.2.1.4. Reprocessing Sephadex G200 fraction pool 1 on Sephadex G200.

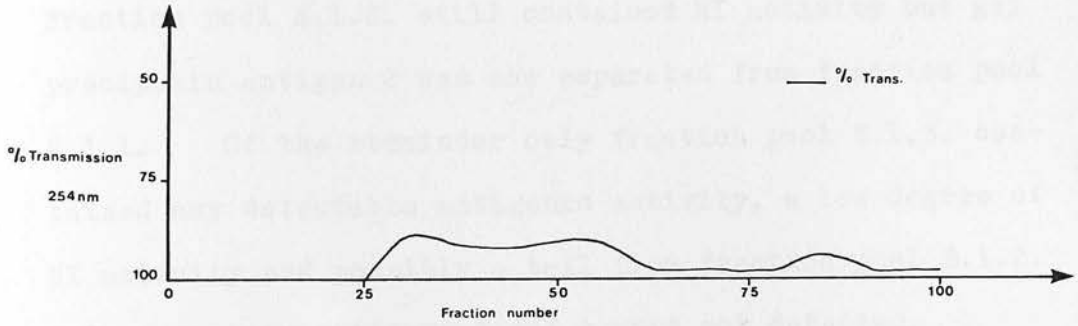
A sample of fraction pool 2.S.1. from Sephadex G200 chromatography was reprocessed on a Sephadex G200 column (Figure 31). The individual sub-fractions were not



Fraction pool		2.S.1.	2.S.2.	2.S.3.	% Recovery
Pooled fractions		35 – 46	47 – 68	69 – 110	
Protein content µg		840 (11·5%)	1080 (14·8%)	440 (6·0%)	32·3%
Carbohydrate content µg		140 (5·1%)	200 (7·3%)	70 (2·5%)	14·9%
HI Titres $\frac{1}{Log_2}$		17	7	3	
Gel precipitins		1(2)	1,2,3,4	2	
Approx. Mol. Wt.	G	10^6	10^5	10^4	
	L	10^5	3×10^4	10^4	

Sample	2·0 ml SE
Protein content	7280 µg
Carbohydrate cont.	2740 µg
Flow rate	3·4 ml/cm²/hr.
Fraction collection	4/hr.

Figure 30. A repeat of the Sephadex G200 chromatography of SE.



Fraction pool	S.1.1.	S.1.2.	S.1.3.	S.1.4.	S.1.5.	% Recovery
Pooled fractions	26-38	39-50	51-63	64-72	73-90	
Protein content µg	760 (18·1%)	1000 (23·8%)	280 (6·6%)	-	180 (4·3%)	52·8%
Carbohydrate content µg	250 (35·7%)	40 (5·7%)	20 (2·9%)	-	50 (7·1%)	51·4%
HI Titres $\frac{1}{Log_2}$	12	6	<2	-	-	
Gel precipitins	1	2	-	-	-	
Approx. mol. wt. G	6×10^5	$1{\cdot}5 \times 10^5$	5×10^4	$1{\cdot}5 \times 10^4$	10^4	
Approx. mol. wt. L	10^5	5×10^4	2×10^4	10^4	10^4	

Sample	10·0ml 2.S.1.
Protein content	4200 µg
Carbohydrate cont.	700 µg
Flow rate	3·4ml/ cm^2/ hr
Fraction collection	4/ hr

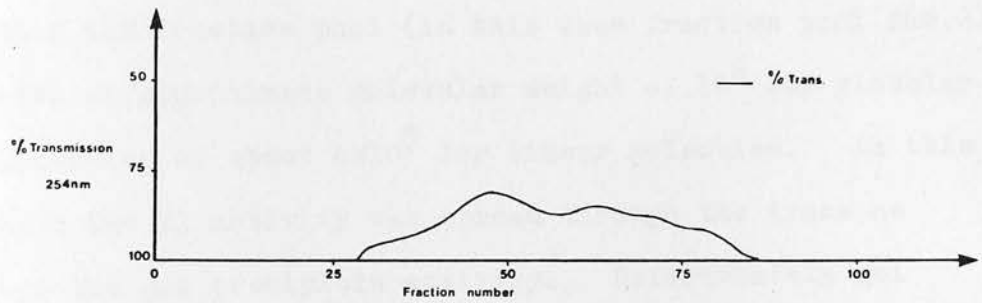
Figure 31. Sephadex G200 chromatography of fraction 2.S.1.

tested for HI activity before pooling and reconcentration. Reprocessing fraction pool 2.S.1. did not result in very distinct peaks. Fraction pool S.1.1. contained most of the HI activity and only gel precipitin antigen 1. Fraction pool S.1.2. still contained HI activity but gel precipitin antigen 2 was now separated from fraction pool S.1.1. Of the remainder only fraction pool S.1.3. contained any detectable antigenic activity, a low degree of HI activity and possibly a tail from fraction pool S.1.2. Gel precipitin antigens 3 and 4 were not detected.

3.3.2.2.1.5. Reprocessing Sephadex G200 fraction pool 2 on Sephadex G200.

A sample of fraction pool 2.S.2. from Sephadex G200 chromatography was reprocessed on a Sephadex G200 column (Figure 32). Reprocessing did not produce very distinct peaks. Fraction pool S.2.1. contained gel precipitin antigens 1 and 2 along with some HI activity. Gel precipitin antigens 1, 2, 3 and 4 were all detected in fraction pool S.2.2. There was also a greater amount of HI activity detected in this fraction pool than in fraction pool S.2.1.

Fraction pool S.2.3. contained a little HI activity and gel precipitin antigen 2, whereas fraction pool S.2.4. contained no detectable antigenic activity.



Fraction pool	S 2.1	S 2.2	S 2.3	S 2.4	% Recovery
Pooled fractions	25–35	36–57	58–66	67–93	
Protein content µg	350 (8·3%)	2370 (56·4%)	500 (11·8%)	–	76·6%
Carbohydrate cont µg	95 (11·8%)	155 (19·4%)	25 (3·1%)	–	34·4%
HI Titres $\frac{1}{\text{Log}_2}$	7	10	2	–	
Gel precipitins	1,2	1,2,3,4	2	–	
Approx. mol. wt. G	$3{\cdot}5 \times 10^5$	10^5	$2{\cdot}0 \times 10^4$	10^4	
Approx. mol. wt. L	$9{\cdot}0 \times 10^4$	$3{\cdot}5 \times 10^4$	$1{\cdot}5 \times 10^4$	10^4	

Sample	8·0ml 2.S.2.
Protein content	4200 µg
Carbohydrate cont.	800 µg
Flow rate	3·4 ml / cm^2 / hr
Fraction collection	4 / hr

Figure 32. Sephadex G200 chromatography of fraction 2.S.2.

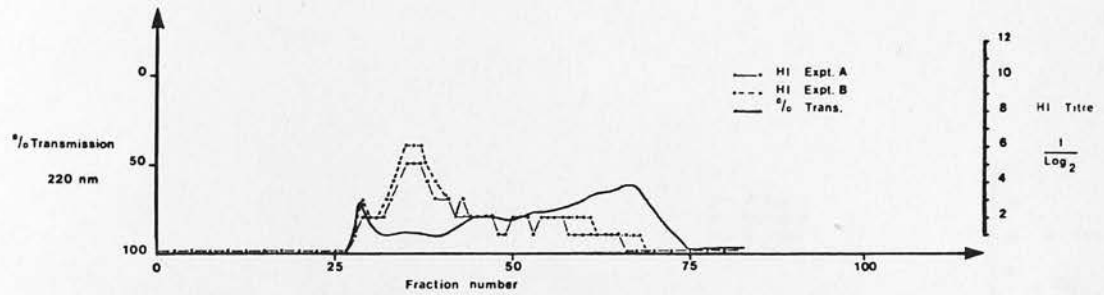
3.3.2.2.2. Fractionation of SE on Sepharose 6B.

A sample of SE was fractionated on Sepharose 6B (Figure 33). The HI activity was again associated with the fraction pool (in this case fraction pool S6B.2) with an approximate molecular weight of 10^6 for globular molecules or about $4x10^5$ for linear molecules. In this case the HI activity was spread through the trace as was the gel precipitin activity. Unfortunately gel precipitin antigens 3 and 4 and much of the HI activity were lost.

3.3.2.2.3. Fractionation of SE on Sepharose 4B.

A sample of SE was fractionated on Sepharose 4B (Figure 34). The graph only shows the HI activity of the sub fractions. There was a small amount of HI activity associated with fraction pool 1.S4B.1. which contained very large molecules or possibly molecules which were aggregated. Most of the HI activity was centred between fraction pools 1.S4B.3. and 1.S4B.4. The molecules in these fraction pools corresponded roughly in size to those fraction pools in which HI activity was found on Sephadex G200 and Sepharose 6B chromatography.

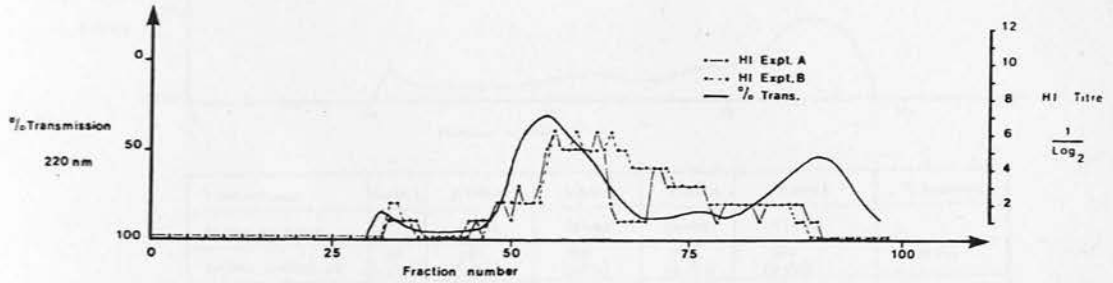
Apart from the small amount of HI activity in fraction pool 1.S4B.1. fractionating SE on Sepharose 4B did not improve the separation as most of the HI activity appeared as one long drawn out peak.



Fraction pool	S6B.1.	S6B.2.	S6B.3.	S6B.4.	S6B.5.	S6B.6.	% Recovery
Pooled fractions	27–31	32–39	40–49	50–57	58–63	64–76	
Protein content µg	220 (6·0%)	195 (5·4%)	360 (9·9%)	380 (10·4%)	350 (9·6%)	150 (4·1%)	45·4%
Carbohydrate cont. µg	185 (13·5%)	160 (11·7%)	200 (14·6%)	255 (18·6%)	95 (6·9%)	260 (19·0%)	84·3%
HI Titres $\frac{1}{Log_2}$	–	2	2	4	–	–	
Gel precipitins	–	–	1	2	2	2	
Approx. mol. wt. G	4×10^6	1.5×10^6	2.5×10^5	3.0×10^4	10^4	$<10^4$	
Approx. mol. wt. L	10^6	4.0×10^5	10^5	2.0×10^4	10^4	$<10^4$	

Sample	1·0ml SE
Protein content	3640 µg
Carbohydrate cont.	1370 µg
Flow rate	4·9 ml/ cm^2/hr
Fraction collection	4/hr.

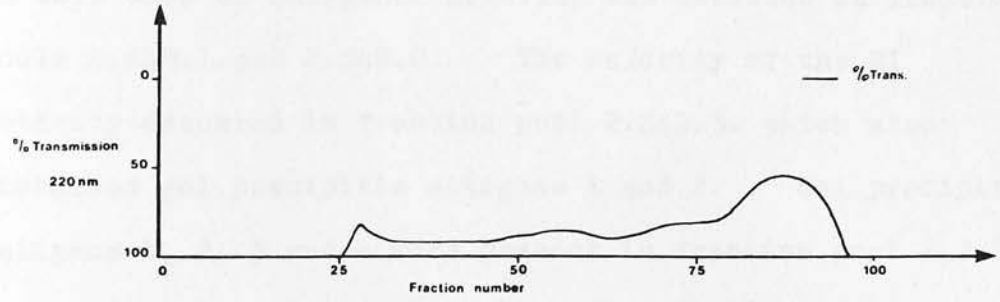
Figure 33. Sepharose 6B chromatography of SE.



Fraction pool		1 S4B 1	1 S4B 2	1 S4B 3	1 S4B 4	1 S4B 5
Pooled fractions		30–36	37–45	46–67	68–80	81–102
Approx. mol. wt.	G	20×10^6	4×10^5	10^6	8×10^4	10^4
	L	5×10^6	2×10^5	4×10^5	4×10^4	10^4

Sample	1·0ml SE
Protein content	3640 µg
Carbohydrate cont.	1370 µg
Flow rate	3·6ml/cm^2/hr
Fraction collection	4/hr

Figure 34. Sepharose 4B chromatography of SE.



Fraction pool	2.S4B.1.	2.S4B.2.	2.S4B.3.	2.S4B.4.	2.S4B.5.	% Recovery
Pooled fractions	25–33	34–48	49–63	64–76	77–96	
Protein content µg	80 (1·5%)	200 (3·8%)	140 (2·7%)	140 (2·7%)	480 (9·1%)	19·8%
Carbohydrate cont µg	100 (5·2%)	100 (5·2%)	40 (2·1%)	–	40 (2·1%)	14·6%
HI Titres $\frac{1}{Log_2}$	–	–	8	6	3	
Gel precipitins	–	–	1,2	1,2 (3,4)	2 (3,4)	
Approx. mol. wt. G	20×10^6	2×10^6	4×10^5	4×10^4	10^4	
Approx. mol. wt. L	5×10^6	10^6	2×10^5	3×10^4	10^4	

Sample	4·0 ml SE
Protein content	5280 µg
Carbohydrate cont.	1930 µg
Flow rate	4·5 ml/ cm^2/ hr
Fraction collection	4/hr

Figure 35. A repeat of the Sepharose 4B chromatography of SE.

A second sample of SE was fractionated by Sepharose 4B (Figure 35), but the individual sub-fractions were not tested for HI activity prior to pooling and reconcentration. In this case no antigenic activity was detected in fraction pools 2.S4B.1. and 2.S4B.2. The majority of the HI activity occurred in fraction pool 2.S4B.3. which also contained gel precipitin antigens 1 and 2. Gel precipitin antigens 1, 2, 3 and 4 were present in fraction pool 2.S4B.4. This fraction pool contained less HI activity than fraction pool 2.S4B.3. Fraction pool 2.S4B.5. contained gel precipitin antigens 2, 3 and 4 along with some HI activity.

The peak for fraction pool 1.S4B.3. was very much larger than the corresponding fraction pool 2.S4B.3., despite the fact that more protein was applied to the Sepharose 4B column for the second fractionation. In addition the total area under the trace was greater for 1.S4B than 2.S4B.

3.3.2.3. Ion exchange chromatography.

3.3.2.3.1. Preliminary batchwise elution.

A batchwise procedure was used to determine whether DE cellulose or CM cellulose was most suitable for use with SE. A relatively small amount of protein was adsorbed onto CM cellulose, as indicated by the large amount left in the supernatant CMS1 (Table 19). Very little protein was eluted from the CM cellulose by either acid or alkaline

Table 19 CM52 and DE52 as ion exchange celluloses for the fractionation of SE.

Type of cellulose & elution buffer used	Protein content		Carbohydrate content		HI titre $\frac{1}{\text{Log}_2}$	Gel Precipitin antigens
	ug/ml	% recovered	ug/ml	% recovered		
CMS1 0.01M Ⓟ pH7.2	87.5	46.7%	22.5	3.8%	7	1,2,3,4.
CMS2 0.1M Na acetate/ acetic acid pH 5.0 + 1.0M NaCl	12.5	6.7%	20.5	3.5%	-	-
CMS3 0.1M Na borate/NaOH pH 9.0 + 1.0M NaCl	4.0	2.1%	24.0	4.1%	-	-
DES1 0.01M Tris/ HCl pH 7.2	25.0	13.3%	47.5	8.1%	-	-
DES2 0.1M Na acetate/ acetic acid pH 5.0 + 1.0M NaCl	37.5	20.0%	7.5	1.3%	6	1,2,3.
DES3 0.1M Tris/ HCl pH 8.9 + 1.0M NaCl	50.0	26.7%	5.0	0.8%	6	1,2,3,4.
Untreated SE	187.5	-	590.0	-	11	1,2,3,4.

Ⓟ = phosphate

buffers of high molarity, as indicated by the low protein content of fractions CMS2 and CMS3 respectively. However, there was very little carbohydrate left in fraction CMS1 and hardly any eluted in fractions CMS2 and CMS3. All the detectable antigenic activity was in fraction CMS1.

Either no antigenically active molecules attach to CM cellulose equilibrated to 0.01M phosphate buffer pH7.2 or, if they do, they are not eluted by either acid or alkaline buffers of high molarity.

In contrast most of the protein was adsorbed onto the DE cellulose equilibrated to 0.01 M Tris/HCl buffer pH 7.2, and eluted by both acid and alkaline buffers of high molarity. Some carbohydrates were not adsorbed onto the DE cellulose as indicated by the relatively large amount of carbohydrate in fraction DES1. Only a small amount of carbohydrate was eluted by the acid and alkaline buffers of high molarity. No antigenic activity was detected in fraction DES1, but both gel precipitin antigens and HI antigens were detected in fractions DES2 and DES3.

The antigenically active molecules in SE were adsorbed onto the DE cellulose equilibrated to 0.01M Tris/HCl buffer pH 7.2 and were eluted by buffers of either acid or alkaline pH and high molarity.

3.3.2.3.2. Gradient elution.

3.3.2.3.2.1. System 1 - Gradient 1 without the level sensor.

A sample of SE was fractionated on a DE column using gradient 1 (Figure 36a), a straight line gradient from 0 - 100 per cent final buffer. The level sensor was not in operation. The saline extract was not separated into distinct peaks (Figure 37). Most of the material was eluted from the column in one large heterogeneous group of molecules. Some material was not adsorbed to the DE cellulose and came off in sub fractions 8 - 20, but this material did not have any detectable antigenic activity.

The molecules containing antigenic activity were eluted in the large second peak at buffer concentrations of between 0.037 M phosphate, 0.27M NaCl and 0.1M phosphate, 0.9M NaCl. For analysis this peak was split into three fraction pools. The HI activity was centred on fraction pool DE1.3. and the gel precipitin activity was spread over fraction pools DE1.2. - DE1.4. It was difficult to detect the gel precipitin antigens because the precipitin lines were faint. The presence of a small amount of HI activity in fraction pools DE1.2. and DE1.4. can be explained as a tailing effect from fraction pool DE1.3., especially as some of the sub-fractions containing HI activity were included in fraction pools DE1.2. and DE1.4.

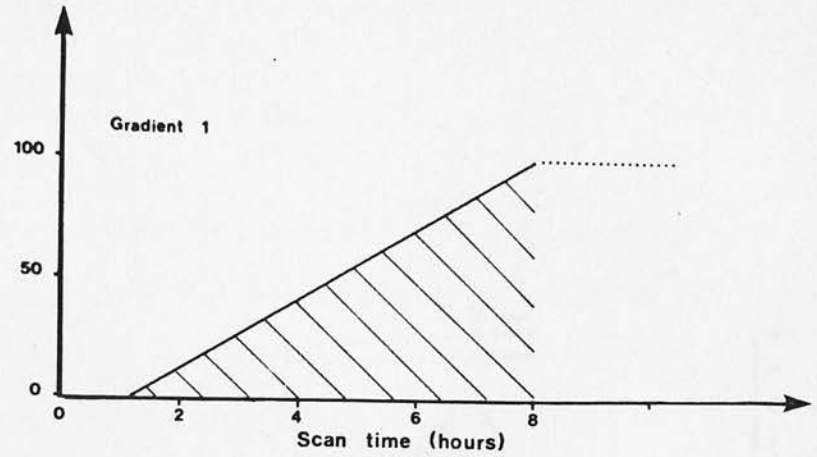


Figure 36a.

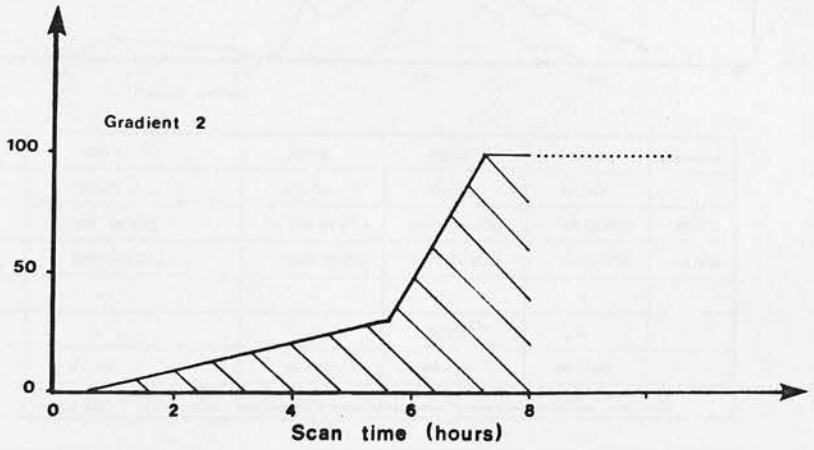


Figure 36b.

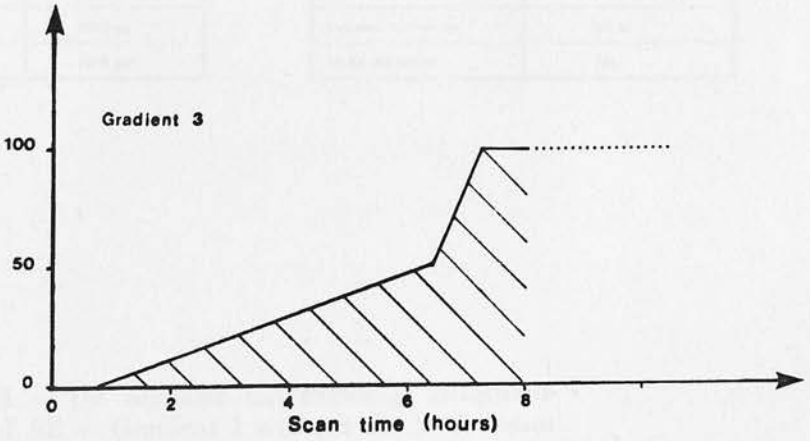
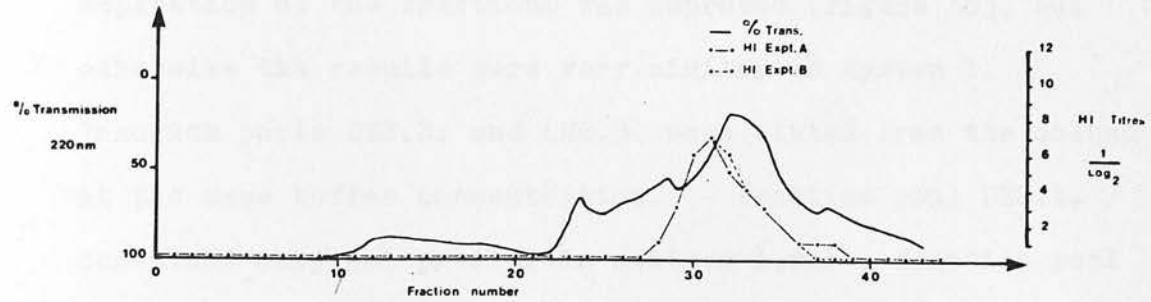


Figure 36c.

Figure 36. The three elution gradients used in ion-exchange chromatography.



Fraction pool	DE1. 1.	DE1. 2.	DE1. 3.	DE1. 4.	%Recovery
Pooled fractions	8 – 20	21 – 28	29 – 36	37 – 43	
Protein content µg	120 (4·0%)	135 (4·5%)	638 (21·0%)	90 (3·0%)	32·3%
Carbohydrate cont. µg	330 (14·3%)	52·5 (2·3%)	37·5 (1·6%)	7·5 (0·3%)	18·5%
HI Titres $\frac{1}{\log_2}$	–	3	11	5	
Gel precipitins	–	1	2 (3,4)	2	
% Final buffer	0 – 30	30 – 56	56 – 80	80 – 100	
Elution molarity	0·010 M (P) 0·000M NaCl – 0·037M (P) 0·270M NaCl	0·037M (P) 0·270M NaCl – 0·061M (P) 0·504M NaCl	0·061M (P) 0·504M NaCl – 0·082M (P) 0·720M NaCl	0·082M (P) 0·720M NaCl – 0·100M (P) 0·900M NaCl	

Sample	15·0 ml SE
Protein content	3015 µg
Carbohydrate content	2310 µg

Flow rate	10·2 ml/cm²/hr
Fraction collection	10/hr
DEAE cellulose	20g

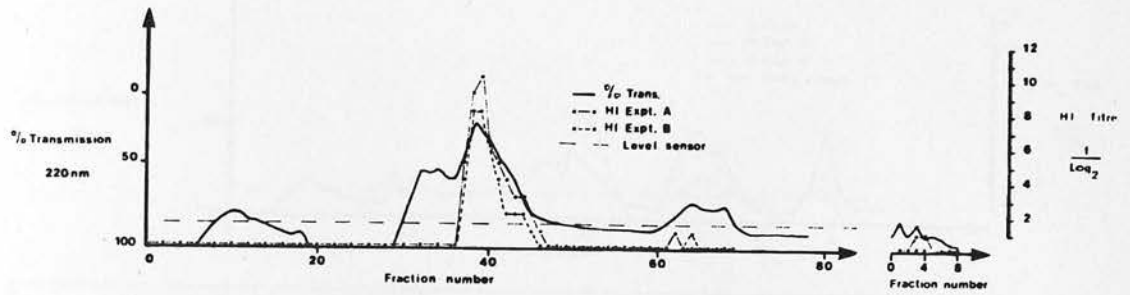
Figure 37. System 1 – DE cellulose ion exchange chromatography of SE – Gradient 1 without the level sensor in operation.

3.3.2.3.2.2. System 2 - Gradient 1 with the level sensor.

When gradient 1 was used with the level sensor the separation of the fractions was improved (Figure 38), but otherwise the results were very similar to system 1. Fraction pools DE2.2. and DE2.3. were eluted from the column at the same buffer concentration. Fraction pool DE2.1. contained only gel precipitin antigen 1,while fraction pool DE2.3. contained both gel precipitin and HI activity. There was antigenic activity associated with fraction pool DE2.4., eluted at a higher buffer concentration and also fraction pool DE2.5. eluted by 0.1M phosphate buffer, 0.9M NaCl pH8.0.

3.3.2.3.2.3. System 3 - Gradient 2 with the level sensor.

Gradient 2 (Figure 36b) was designed to attempt further separation of fraction pools DE2.2. and DE2.3. eluted by system 2. This system separated SE into many more peaks (Figure 39). The main results were that fraction pool DE3.3. contained gel precipitin antigens 1, 3 and 4 but no detectable HI activity and that the HI antigens were split among three separate fraction pools. In addition to HI activity, fraction pools DE3.4., DE3.5. and DE3.6. all contained gel precipitin antigen 2, although this was strongest in fraction pool DE3.5. The fraction pool from system 2 which was eluted with 0.055M

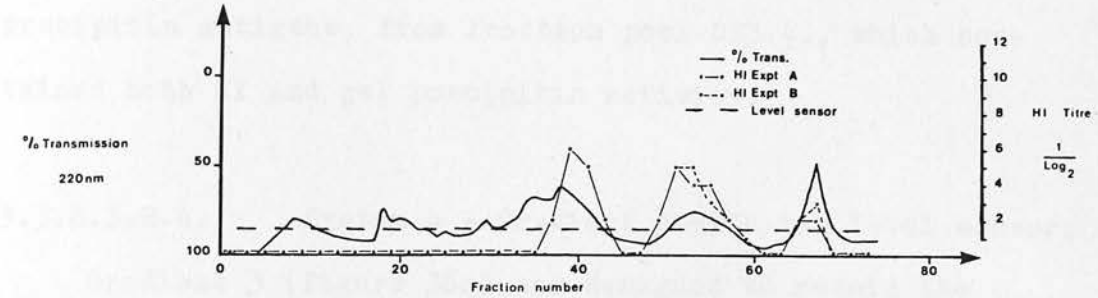


Fraction pool	DE2.1.	DE2.2.	DE2.3.	DE2.4.	DE2.5.	% Recovery
Pooled fractions	1 – 29	30 – 36	37 – 55	56 – 78	1 – 8	
Protein content µg.	340 (8·5%)	350 (8·8%)	400 (10·0%)	120 (3·0%)	120 (3·0%)	31·0%
Carbohydrate content µg.	590 (19·2%)	20 (0·7%)	–	–	10 (0·3%)	20·2%
HI Titre $\frac{1}{\text{Log}_2}$	–	–	11	4	3	
Gel precipitins	–	1	2 (1,3,4)	2	–	
% Final buffer	0	25	25	50·25	100	
Elution molarity	0·010 M (P) 0·000 M NaCl	0·032 M (P) 0·225 M NaCl		0·055 M (P) 0·452 M NaCl	0·100 M (P) 0·900 M NaCl	

Sample	20·0 ml SE
Protein content	4020 µg
Carbohydrate content	3081 µg

Flow rate	10·2 ml/cm²/hr
Fraction collection	10/hr
DEAE cellulose	20g

Figure 38. System 2 – DE cellulose ion exchange chromatography of SE – Gradient 1 with the level sensor in operation.



Fraction pool	DE3.1.	DE3.2.	DE3.3.	DE3.4.	DE3.5.	DE3.6.	% Recovery
Pooled fractions	1 – 16	17 – 27	28 – 36	37 – 46	47 – 61	62 – 71	
Protein content µg.	100 (2·9%)	–	120 (3·0%)	60 (1·5%)	120 (3·0%)	–	9·9%
Carbohydrate content µg.	70 (2·3%)	72 (2·3%)	72 (2·3%)	50 (1·6%)	25 (6·8%)	20 (0·7%)	10·0%
HI Titre $\frac{1}{Log_2}$	–	–	–	8	8	5	
Gel precipitins	–	–	1 (3,4)	2	2	2	
% Final buffer	0	7	20	22	27	100	
Elution molarity	0·010 M Ⓟ 0·000 M NaCl	0·016 M Ⓟ 0·063 M NaCl	0·028 M Ⓟ 0·180 M NaCl	0·030 M Ⓟ 0·198 M NaCl	0·034 M Ⓟ 0·243 M NaCl	0·100 M Ⓟ 0·900 M NaCl	

Sample	20·0 ml SE
Protein content	4020 µg
Carbohydrate content	3080 µg

Flow rate	10·2 ml / cm^2 / hr
Fraction collection	10 / hr
DEAE cellulose	20g

Figure 39. System 3 – DE cellulose ion exchange chromatography of SE – Gradient 2 with the level sensor in operation.

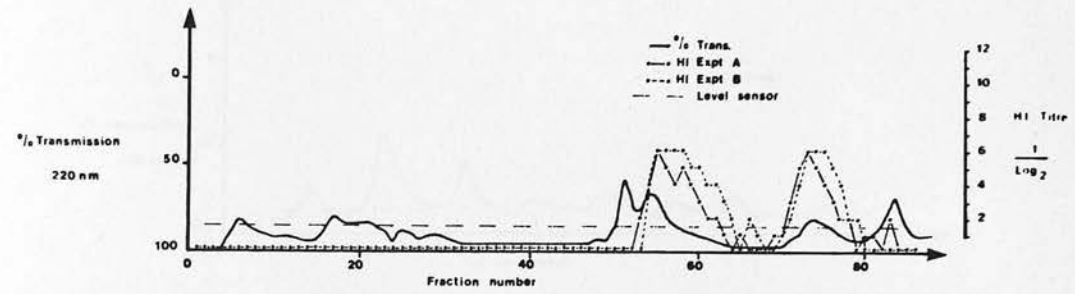
phosphate buffer, 0.45M NaCl pH 8.0 was most probably eluted from this column with the 0.1M phosphate buffer, 0.9M NaCl pH8.0 fraction. Unfortunately this gradient did not separate fraction pool DE3.3., which contained only gel precipitin antigens, from fraction pool DE3.4., which contained both HI and gel precipitin activity.

3.3.2.3.2.4. System 4 - Gradient 3 with the level sensor.

Gradient 3 (Figure 36c) was designed to retain the separation in the fractions eluted at lower molarities using system 3 but also to attempt to separate the fraction pool which was eluted by 0.055M phosphate buffer, 0.45M NaCl pH8.0 using system 2. The results (Figure 40) were very similar to those obtained with system 3. The fraction pool which was eluted by 0.055M phosphate buffer, 0.45M NaCl pH8.0 in system 2 was eluted with the 0.1M phosphate buffer, 0.9M NaCl pH8.0. Fraction pool DE4.4. which contained only gel precipitin antigen 1 was not separated from fraction pool DE4.5. which contained both HI antigens and gel precipitin antigen 2. Gel precipitin antigens 3 and 4 were not detected.

3.3.2.3.2.5. System 5 - System 4 with a reduced amount of DE cellulose.

System 5 resulted in improved separation of the various fraction pools (Figures 41 and 42). Eight separate fraction pools were eluted from the column. In both cases only fraction pool DE5.2. appeared to contain more than one peak.

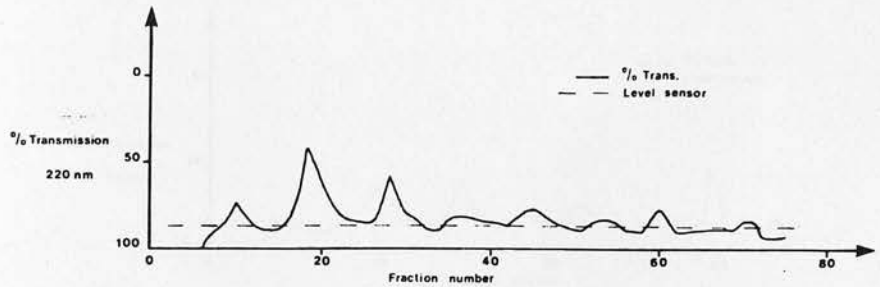


Fraction pool	DE4.1.	DE4.2.	DE4.3.	–	DE4.4.	DE4.5.	DE4.6.	DE4.7.	DE4.8.	% Recovery
Pooled fractions	4–14	15–23	24–32	–	48–52	53–60	61–69	70–80	81–88	
Protein content µg	40 (1·5%)	260 (9·8%)	10 (0·4%)	–	40 (1·5%)	80 (3·0%)	130 (4·9%)	110 (4·2%)	60 (2·3%)	27·6%
Carbohydrate content µg	55 (5·7%)	80 (8·3%)	35 (3·6%)	–	70 (7·3%)	115 (12·0%)	15 (1·6%)	25 (2·6%)	24 (2·5%)	43·6%
HI Titre $\frac{1}{\text{Log}_2}$	–	–	–	–	–	6	4	3	1	
Gel precipitins	–	–	–	–	1	2	2	2	–	
% Final buffer	0	8	10	–	20	20	24	28	100	
Elution molarity	0·010M P 0·000M NaCl	0·017M P 0·072M NaCl	0·019M P 0·090M NaCl	–	0·028M P 0·180M NaCl		0·032M P 0·215M NaCl	0·035 M P 0·252 M NaCl	0·100 M P 0·900M NaCl	

Sample	2·0ml S.E.
Protein content	2480 µg
Carbohydrate content	960 µg

Flow rate	10·2 ml/ cm²/ hr
Fraction collection	10/ hr
DEAE cellulose	20g

Figure 40. System 4 – DE cellulose ion exchange chromatography of SE – Gradient 3 with the level sensor in operation.

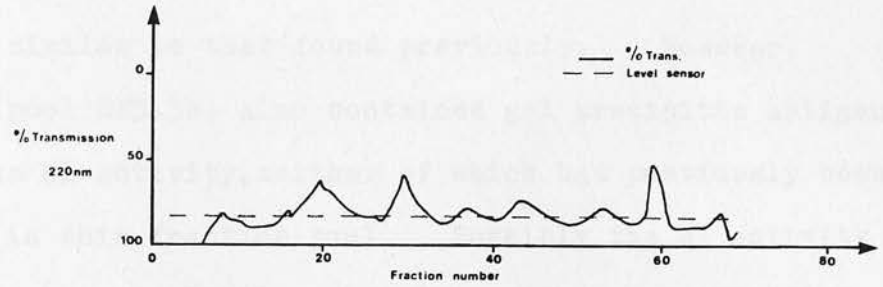


Fraction pool	DE5. 1a.	DE5. 2a.	DE5. 3a.	DE5. 4a.	DE5. 5a.	DE5. 6a.	DE5. 7a.	DE5. 8a.	% Recovery
Pooled fractions	7–14	15–24	25–33	34–41	42–50	51–57	58–62	69–71	
Protein content µg	180 (3·0%)	310 (5·2%)	160 (2·7%)	80 (1·3%)	216 (3·5%)	50 (0·8%)	0	20 (0·3%)	16·8%
Carbohydrate content µg	95 (5·4%)	185 (10·5%)	315 (17·9%)	17 (1·0%)	60 (3·4%)	12·5 (0·7%)	0	180 (10·2%)	49·1%
HI Titre $\frac{1}{Log_2}$	–	–	7	6–7	4	3	1	–	
Gel precipitins	–	–	1,2	2 (1,3,4)	2	2	–	–	
%Final buffer	7·5	16	20	28	35	50	100	100	
Elution molarity	0·017M (P) 0·068M NaCl	0·024M (P) 0·144M NaCl	0·028M (P) 0·180M NaCl	0·035M (P) 0·252M NaCl	0·042M (P) 0·315M NaCl	0·055M (P) 0·450M NaCl	0·100M (P) 0·900M NaCl		

Sample	2·0ml SE
Protein content	6000 µg
Carbohydrate content	1760 µg

Flow rate	10·2ml/ cm^2/ hr
Fraction collection	4/ hr
DEAE cellulose	15g

Figure 41. System 5 – DE cellulose ion exchange chromatography of SE using a reduced quantity of cellulose – Gradient 4 with the level sensor in operation.



Fraction pool	DE5.1b.	DE5.2b.	DE5.3b.	DE5.4b.	DE5.5b.	DE5.6b.	DE5.7b.	DE5.8b.	%Recovery
Pooled fractions	7–13	14–26	27–34	35–40	41–49	50–57	58–60	66–67	
Protein content µg	290 (4·8%)	340 (5·7%)	80 (1·3%)	280 (4·7%)	340 (5·7%)	90 (1·5%)	60 (1·0%)	80 (1·3%)	26%
Carbohydrate content µg	105 (5·7%)	240 (13·6%)	90 (5·1%)	180 (10·2%)	185 (10·5%)	96 (5·1%)	65 (3·6%)	135 (7·7%)	61·5%
HI Titre $\frac{1}{\log_2}$	–	–	–	4	3–4	2–3	–	–	
Gel precipitins	–	–	1	2 (3,4)	2	2	–	–	
% Final buffer	7·5	18	24	32	42	88	100	100	
Elution molarity	0·017M (P) 0·068M NaCl	0·026M (P) 0·162M NaCl	0·032M (P) 0·216M NaCl	0·036M (P) 0·285M NaCl	0·048M (P) 0·378M NaCl	0·091M (P) 0·810M NaCl	0·100M (P) 0·900M NaCl		

Sample	2·0ml SE
Protein content	6000 µg
Carbohydrate content	1760 µg

Flow rate	10·2ml / cm^3 / hr
Fraction collection	4 / hr
DEAE cellulose	15g

Figure 42. A repeat of system 5 – DE cellulose chromatography of SE using a reduced quantity of cellulose – Gradient 4 with the level sensor in operation.

The overall percentage of material recovered was only slightly improved on previous fractionations.

As with previous results the first two fraction pools contained no detectable antigenic activity. Gel precipitin antigen 1 was present in fraction pool DE5.3a., a result similar to that found previously. However, fraction pool DE5.3b. also contained gel precipitin antigen 2 and some HI activity, neither of which had previously been detected in this fraction pool. Possibly the HI activity was caused by very labile molecules which had previously been denatured. Equally the HI activity may have been associated with the presence of gel precipitin antigen 2, an antigen usually associated with HI activity. In previous experiments fraction pools corresponding to DE5.3. and DE5.4. were eluted as a double peak with the second part containing HI activity. In both experiments with system 5 these fraction pools were eluted as separate peaks.

Gel precipitin antigen 2 and possibly antigens 3 and 4 were present in fraction pool DE5.4a. Gel precipitin antigen 1 may also have been present in fraction pool DE5.4b. Fraction pool DE5.4a. and b. also contained HI activity as did fraction pools DE5.5a. and b. and DE5.6a. and b. These fraction pools also contained gel precipitin antigen 2. Fraction pool DE5.7a. contained no detectable antigenic activity but a very small amount of HI activity was detected in fraction pool DE5.7b. Fraction pools DE5.8a. and b. contained no detectable antigenic activity. This was an

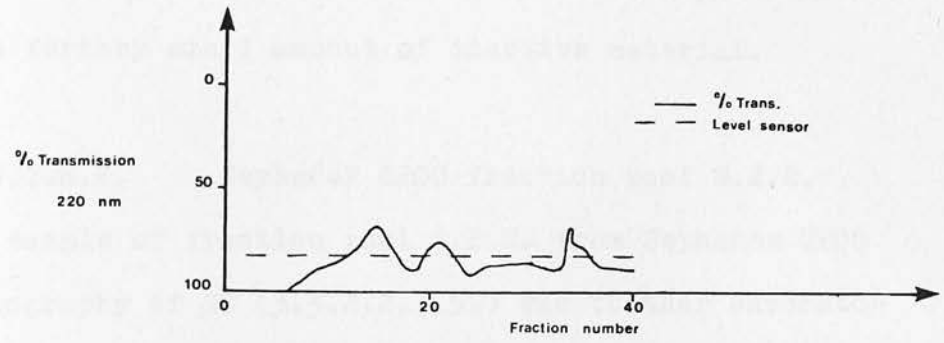
interesting fraction pool as it was eluted some time after the 0.1M phosphate buffer, 0.9M NaCl pH8.0 fraction pool and must have been composed of molecules which were strongly adsorbed onto the DE cellulose.

3.3.2.3.2.6. Ion exchange chromatography of Sephadex G200 fractionated extracts of SE.

3.3.2.3.2.6.1. Sephadex G200 fraction pool S.1.1.

A sample of fraction pool S.1.1. from Sephadex G200 chromatography of SE (3.3.2.2.1.3.) was fractionated on a DE cellulose column using gradient 3 and the level sensor (Figure 43). Fraction pool S.1.1. contained HI activity and gel precipitin antigen 1 although gel precipitin antigen 2 was also present at low concentration.

The extract was split into 5 fraction pools although the last fraction pool appeared to be a double peak. These results were rather different from the fractionation of the untreated SE (3.3.2.3.2.5.). Unfortunately the individual subfractions were not checked for HI activity but HI activity was present in fraction pools DE6.1. - DE6.4. Fraction pools DE5.1. and DE5.2. obtained on fractionation of the untreated SE (3.3.2.3.2.5.) were absent in this instance. Fraction pools DE5.1. and DE5.2. may have been composed of small molecules which were removed by filtration through Sephadex G200, but this did not explain the absence of fraction pool DE5.3. which contained the large gel precipitin antigen 1 alone. Clearly this



Fraction pool	DE6. 1.	DE6. 2.	DE6. 3.	DE6. 4.	DE6. 5.	% Recovery
Pooled fractions	7–11	12–18	19–24	25–33	34–37	
Protein content µg	70 (4·2%)	500 (29·8%)	270 (16·1%)	140 (8·3%)	40 (2·4%)	60·8%
Carbohydrate content µg	35 (12·5%)	25 (8·9%)	24 (8·5%)	20 (7·1%)	1 (0·4%)	37·4%
HI Titre $\frac{1}{\text{Log}_2}$	12	12	6	2	–	
Gel precipitins	–	1,2	1,2	1,2	–	
% Final buffer	7·5	20	28	32	80	
Elution molarity	0·017 M (P) 0·068 M NaCl	0·028 M (P) 0·180 M NaCl	0·035 M (P) 0·252 M NaCl	0·039 M (P) 0·288 M NaCl	0·082 M (P) 0·720 M NaCl	

Sample	4·0 ml 2.S.1.
Protein content	1680 µg
Carbohydrate content	280 µg

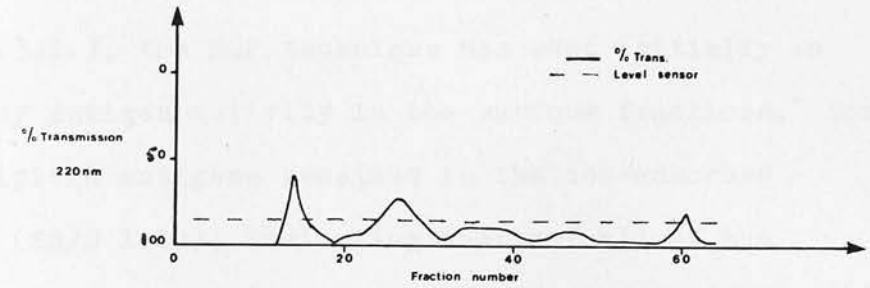
Flow rate	10·2 ml / cm^2 / hr
Fraction collection	4 / hr
DEAE cellulose	15g

Figure 43. DE cellulose ion exchange chromatography of fraction 2.S.1. using system 5 – Gradient 4 with the level sensor in operation.

fractionation was sub-optimal, perhaps molecular aggregation and complex formation had occurred after Sephadex G200 purification of the untreated SE and had affected the net charge on the particles. Nevertheless this procedure removed a further small amount of inactive material.

3.3.2.3.2.6.2. Sephadex G200 fraction pool S.2.2.

A sample of fraction pool S.2.2. from Sephadex G200 chromatography of SE (3.3.2.2.1.5.) was further chromatographed on DE cellulose as described (3.3.2.3.2.6.1.). Again fraction pools corresponding to DE5.1. and DE5.2. were not present (Figure 44). This suggested that these molecules were removed by Sephadex G200 fractionation. Fraction pool DE7.1. may have corresponded to fraction pool DE5.3. from chromatography of the crude SE (3.3.2.3.2.5.) since this fraction pool was eluted at approximately the same buffer concentration. It contained gel precipitin antigen 1 but also gel precipitin antigen 2 and a little HI activity. Fraction pool DE7.2. contained all the gel precipitin antigens 1, 2, 3 and 4 along with a small amount of HI activity, whereas fraction pool DE7.3 only contained gel precipitin antigens 1 and 2. Apart from the absence of fraction pools corresponding to fraction pools DE5.1. and DE5.2. this fractionation produced no new or unusual fraction pools.



Fraction pool	DE7.1	DE7.2.	DE7.3.	DE7.4.	DE7.5.	DE7.6.	% Recovery
Pooled fractions	9–19	20–32	33–42	43–50	51–58	59–65	
Protein content µg	2·5 (0·1%)	260 (12·0%)	90 (4·2%)	7·5(0·4%)	2·5(0·1%)	80(3·7%)	20·5%
Carbohydrate content µg	50 (12·5%)	35 (8·8%)	35 (8·8%)	12·5(3·1%)	35 (8·8%)	25(6·3%)	48·3%
HI Titre $\frac{1}{Log_2}$	1	5	–	–	–	–	
Gel precipitins	1,2	1,2,3,4	1,2	–	–	–	
% Final buffer	18	27	32	55	90	100	
Elution molarity	0·026 M (P) 0·162 M NaCl	0·034 M (P) 0·243 M NaCl	0·039 M (P) 0·288 M NaCl	0·060 M (P) 0·495 M NaCl	0·091 M (P) 0·810 M NaCl	0·100 M (P) 0·900 M NaCl	

Sample	4·0ml 2.S.2.
Protein content	2160 µg
Carbohydrate content	400 µg

Flow rate	8·2ml/ cm^2/ hr
Fraction collection	4 / hr
DEAE cellulose	15g

Figure 44. DE cellulose ion exchange chromatography of fraction 2.S.2. using system 5 – Gradient 4 with the level sensor in operation.

3.3.3. Immunoadsorption.

3.3.3.1. Preliminary experiments.

3.3.3.1.1. Experiment 1a.

In this preliminary experiment on immunoadsorption (2.6.1.9.3.1.), the MGP technique was used initially to detect any antigen activity in the various fractions. Some gel precipitin antigens remained in the non-adsorbed material (SN/S 1-10), indicating that not all of the antigen was adsorbed onto the immunoadsorbents (Table 20). However, some gel precipitin antigens were eluted with the change of buffer. Gel precipitin antigens were found in nearly all of the fractions but in two cases they were not detected (-) and in another two cases the gel precipitin lines were very faint (+?).

Table 20 Gel precipitin antigens in extracts of SE purified by immunoadsorption.

Immuno-adsorbent	Fraction code no.	Non-adsorbed material	Fraction code no.	Elution buffer	Eluted material
E25	SN1	+	SN11	glycine/HCl pH2.8	+
	SN2	+	SN12	5M NaI pH9.0	+
	SN3	+	SN13	2.5M NaI pH9.0	+
	SN4	+	SN14	5M $MgCl_2$ pH7.0	+
	SN5	+	SN15	2.5M $MgCl_2$ pH7.0	+
E24	SN6	+?	SN16	glycine/HCl pH2.8	+?
	SN7	+	SN17	5M NaI pH9.0	+
	SN8	+	SN18	2.5M NaI pH9.0	+
	SN9	+	SN19	5M $MgCl_2$ pH7.0	-
	SN10	-	SN20	2.5M $MgCl_2$ pH7.0	+

\+ gel precipitin antigens present

\- gel precipitin antigens absent

Unfortunately when this experiment was repeated, no gel precipitin antigens were detected in any of the fractions.

Although there was HI activity in the non-adsorbed material (Table 21), none was detected in the fractions eluted from the immunoadsorbents despite checking on two separate

Table 21 Haemagglutinins in extracts of SE purified by immunoadsorption.

Immuno-adsorbent	Fraction code no.	Non-adsorbed material HI titre $\frac{1}{\text{Log } 2}$ Expt.A	Expt.B	Fraction code no.	Elution buffer	Eluted material HI titre $\frac{1}{\text{Log}_2}$ Expt.A	Expt.B
E25	SN1	6	7	SN11	glycine/HCl pH2.8	-	-
	SN2	10	6	SN12	5M NaI pH9.0	-	-
	SN3	7	7	SN13	2.5M NaI pH9.0	-	-
	SN4	6	9	SN14	5M $MgCl_2$ pH7.0	-	-
	SN5	7	9	SN15	2.5M $MgCl_2$ pH7.0	-	-
E24	SN6	9	9	SN16	Glycine/HCl pH2.8	-	-
	SN7	6	7	SN17	5M NaI pH9.0	-	-
	SN8	7	8	SN18	2.5M NaI pH9.0	-	-
	SN9	8	6	SN19	5M $MgCl_2$ pH7.0	-	-
	SN10	8	6	SN20	2.5M $MgCl_2$ pH7.0	-	-
Untreated SE		11	11				

occasions (A and B). The HI activity was very low therefore, in this case the HI titres were expressed as $\frac{1}{\text{Log}_2}$ of the dilution of antigen at which there was complete haemagglutination of the tanned and antigen coated red blood cells.

3.3.3.1.2. Experiment 1b.

In this experiment the ELISA inhibition technique was used to detect antigenic activity in the fractions eluted from the immunoadsorbents. The protein content in the fractions could not be estimated because the fractions had been made up in 5 per cent albumin for stabilisation (2.6.1.9.3.1.). Two sera (E21 and E24) were used separately to absorb the antigen activity in the fractions.

The ELISA readings for serum E21 were inhibited by the fractions prepared from both immunoadsorbents (Table 22) but serum E24 was possibly not inhibited. Serum E24 may have contained too high a concentration of antibodies compared to the amount of antigen in the fractions. The presence of the 5 per cent albumin may also have interfered with these results as the ELISA readings for the 5 per cent albumin mixed with E24 were much lower than the saline control i.e. E24 mixed with saline.

The 5 per cent albumin did not markedly effect the ELISA readings for serum E21. Not all of the antigen was adsorbed onto the immunoadsorbent, as there was still some antigen activity in the non-adsorbed material.

Table 22 The ELISA inhibition technique used to detect antigens in extracts of SE purified by immunoadsorption.

Immunoadsorbent	Serum used for absorption	ELISA readings* at 400 nm for each antigen fraction and the elution buffer used				
		SN1 glycine/ HCl pH2.8	SN2 5M NaI pH9.0	SN3 non-adsorbed material	Saline control	5% egg albumin
E25	E21	0.26, 0.24	0.26, 0.29	0.31, 0.30	0.40, 0.38	0.38, 0.36
	E24	0.51, 0.49	0.35, 0.46	0.43, 0.43	0.56, 0.65	0.49, 0.44
E24	E21	0.25, 0.29	0.18, 0.17	0.10, 0.08	0.40, 0.38	0.38, 0.36
	E24	0.47, 0.47	0.44, 0.46	0.26, 0.25	0.56, 0.65	0.49, 0.44

* Corrected to 2 d.p.

3.3.3.2. Batchwise immunoadsorption - experiment 2.

Albumin was not used for stabilisation in this experiment (2.6.1.9.3.2.), therefore, it was possible to estimate the protein content of the fractions (Table 23) using the method of Warburg & Christian (1941).

Table 23 The protein content of fractions of SE purified by immunoadsorption.

Fraction	Immunoadsorbent	Elution buffer	Protein content µg/ml
SN1	Normal bovine	glycine/HCl pH2.8	43.35
SN2	serum (NBS)	5M NaI pH9.0	57.80
SN3	T.ovis infected	glycine/HCl pH2.8	82.80
SN4	sheep serum (TO)	5M NaI pH9.0	43.40
SN5	Pooled serum from T.saginata	glycine/HCl pH2.8	124.90
SN6	infected cattle (PO)	5M NaI pH9.0	80.80
SN7	-	non-adsorbed material	65.00

Every fraction contained some protein indicating that material had been eluted from the immunoadsorbents. The fraction containing the most protein was SN5, which was eluted from the immunoadsorbent prepared from serum from T. saginata infected cattle. It was this fraction and also SN6 which were expected to contain the 'specific' components of T. saginata.

Fractions SN1 - 7 were used to coat ELISA tubes at protein concentrations of 2.0 µg/ml (Table 24) and 0.5 µg/ml (Table 25). The ELISA readings were all corrected for the saline control reading. The full data for this experiment is given in appendix 3a and appendix 3b. Of the two fractions containing 'specific' T. saginata components, SN6 gave the highest ELISA readings but the ELISA readings were not as high as those obtained with the untreated SE. ELISA tubes coated with SN5 gave remarkably low ELISA readings. Positive serum E25, although giving relatively high ELISA readings with untreated SE, gave very low readings with the purified extracts. Possibly serum E25 did not contain many specific antibodies. The 'non-specific' reading for the bovine serum from the slaughter house was reduced,but not completely eliminated,when SN6 was used as the coating antigen in the ELISA technique.

Table 24 The ELISA technique using fractions of SE purified by immunoadsorption.

Fraction code numbers (Table 22)	$\bar{x}$ ELISA readings* at 400 nm for various sera				
	E23	E25	PO	Pool N8 and N9	Pool N14 and N15
SN1	0.033	0.005	0.023	0.015	0.025
SN2	0.058	0.011	0.035	0.008	0.013
SN3	0.030	0.010	0.035	0.020	0.050
SN4	0.015	0.010	0.040	0.040	0.025
SN5	0.048	0.010	0.073	0.008	0.018
SN6	0.500	0.085	0.405	0.033	0.088
SN7	0.133	0.015	0.100	0.005	0.025
Untreated SE	0.588	0.573	0.610	0.065	0.185

* Corrected to 3d.p.

Table 25 The ELISA technique using fractions of SE purified by immunoadsorption.

Fraction code numbers (Table 22)	$\bar{x}$ ELISA readings* at 400 nm for various sera				
	E23	E25	PO	Pool N8 and N9	Pool N14 and N15
SN1	0.013	0.020	0.025	0.000	0.005
SN2	0.008	0.006	0.018	0.002	0.000
SN3	0.007	0.005	0.020	0.000	0.007
SN4	0.060	0.008	0.058	0.050	0.033
SN5	0.028	0.003	0.035	0.005	0.010
SN6	0.190	0.048	0.193	0.030	0.025
SN7	0.065	0.008	0.063	0.075	0.018
Untreated SE	0.293	0.410	0.425	0.035	0.123

* Corrected to 3d.p.

3.3.3.3. Immunoadsorption chromatography.

A sample of SE was purified by first passing it through an immunoadsorption column composed of insolubilised whole normal bovine serum (N14 and N15). Fraction F1, was eluted with the break through peak (Figure 45) and corresponded to molecules which were not adsorbed onto the immunoadsorbent. Fraction F2 was eluted with 0.1 M glycine/HCl buffer, pH 2.8 containing 2.5 M NaCl. This fraction was composed of the molecules which were adsorbed onto the normal bovine serum immunoadsorbent.

Fraction F1 was then applied to another column (Figure 46) packed with an immunoadsorbent prepared from a 40 per cent ammonium sulphate precipitate of pooled serum from cattle orally infected with T. saginata eggs (2.6.1.9. 3.2.). Fraction F3 eluted with the breakthrough peak contained material which was not adsorbed onto the immunoadsorbent. Fraction F4, which contained the 'specific' T. saginata antigen was eluted with the 0.1 M glycine/HCl buffer, pH 2.8 containing 2.5 M NaCl.

The protein content of the various fractions was estimated by the Folin technique and the carbohydrate content by the anthrone reaction (Table 26).

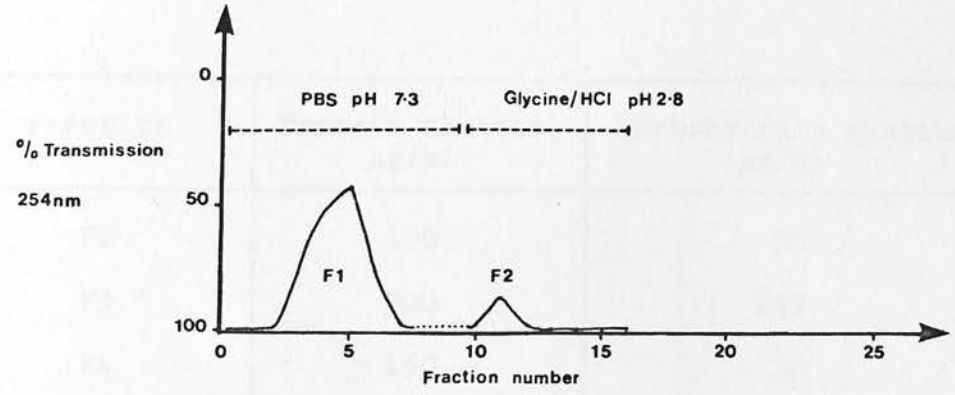


Figure 45. Immunoadsorption chromatography of SE using a chromatographic column packed with polymerised normal bovine serum.

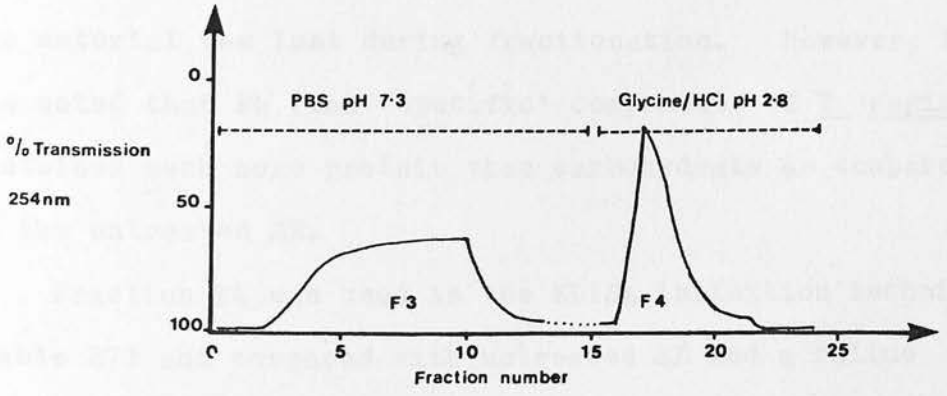


Figure 46. Immunoadsorption chromatography of fraction F1 (Figure 45) using a chromatographic column packed with polymerised serum globulin extracted from the serum of cattle given experimental oral infections of T. saginata eggs.

Table 26 The protein and carbohydrate content of fractions of SE purified by immuno-adsorption.

Fraction	Protein content µg/ml	Carbohydrate content µg/ml
F2	150	55
F3	500	240
F4	150	30
Untreated SE	750	550

The fractions had been concentrated to twice their original sample volume, therefore, approximately half of the material was lost during fractionation. However, it was noted that F4 (the 'specific' components of T. saginata) contained much more protein than carbohydrate as compared to the untreated SE.

Fraction F4 was used in the ELISA inhibition technique (Table 27) and compared with untreated SE and a saline control i.e. serum plus saline instead of a test antigen. Fraction F4 inhibited the ELISA readings for all of the sera but not to such an extent as the untreated SE. The full data for this experiment is given in appendix 3c. In this case serum E24 gave abnormally low ELISA readings as compared with the other positive sera.

Table 27 The ELISA inhibition technique using fraction F4 purified by immunoadsorption.

Serum	$\bar{x}$ ELISA readings* at 400 nm		
	F4	Unpurified antigen	Saline control
E23	0.460	0.130	0.750
E24	0.295	0.195	0.340
E25	0.335	0.110	0.640
PO	0.283	0.080	0.595
Pooled N8 and N9	0.020	0.020	0.090
Pooled N14 and N15	0.003	0.045	0.180

* Corrected to 3d.p.

Table 28 The ELISA technique using fraction F4 purified by immunoadsorption.

Serum	$\bar{x}$ ELISA readings* at 400 nm	
	F4	Unpurified antigen
E23	0.305	0.825
E24	0.405	0.750
E25	0.225	0.595
PO	0.205	0.505
Pool N8 and N9	0.030	0.115
Pool N14 and N15	0.050	0.225

* Corrected to 3d.p.

Fraction F4 was next used to coat ELISA tubes at a protein concentration of 1 µg/ml. The readings obtained using the conventional ELISA procedure were compared with those obtained with untreated SE, also coated onto the ELISA tubes at a protein concentration of 1 µg/ml (Table 28). The ELISA readings for F4 were lower than the untreated SE.

The 'non-specific' readings in the normal bovine sera were reduced. The ELISA reading for the pooled slaughter house sera (N14 and N15) was reduced from 30 per cent of the standard serum reading (E24) to 12.3 per cent. The ELISA readings for the pooled experimental control sera (N8 and N9) were reduced from 15.3 per cent to 7.4 per cent. The full data for this experiment is given in appendix 3d.

Finally, fraction F4 was tested for gel precipitin antigens and, as can be seen (Figure 47), the fraction contained at least three relatively strong precipitin antigens which showed a reaction of identity with one of the precipitin antigens present in SE.

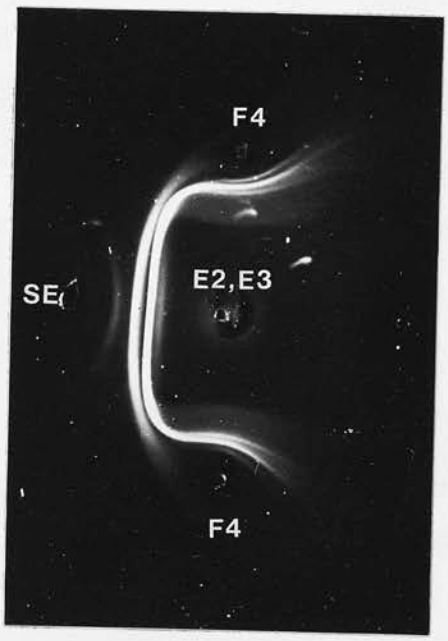


Figure 47. Gel precipitin antigens in fraction F4 of SE purified by immunoadsorption.

3.4. Serology.

3.4.1. Various extracts of T. saginata used in serological techniques.

3.4.1.1. Different antigen preparations for coating ELISA tubes.

3.4.1.1.1. T. saginata larval antigens.

T. saginata cyst fluid and a saline extract of T. saginata cysticercal scolices and membranes were tested along with SE for antigenic activity in the ELISA technique. Sets of 2 ELISA tubes were used and the results expressed as the mean of the ELISA readings minus the mean of the saline control readings (Figure 48). Two different positive control sera were used, one from a calf orally infected with T. saginata eggs (E25) and the other from a calf given a series of i/m injections with a saline extract of T. saginata proglottids (E24). It was decided that 6 µg/ml of protein was a suitable concentration of scolex and membrane extract to coat the ELISA tubes whereas 0.6 µg/ml protein was selected for the cyst fluid. The lower protein concentration used for the cyst fluid did not have any effect on the background readings as the saline controls remained low. The full data for this experiment is given in appendix 4a.

Low readings were obtained with the serum from the calf given a series of i/m injections, when cyst fluid was used as antigen, whereas the serum from the orally infected calf gave similar readings with all three antigen extracts.

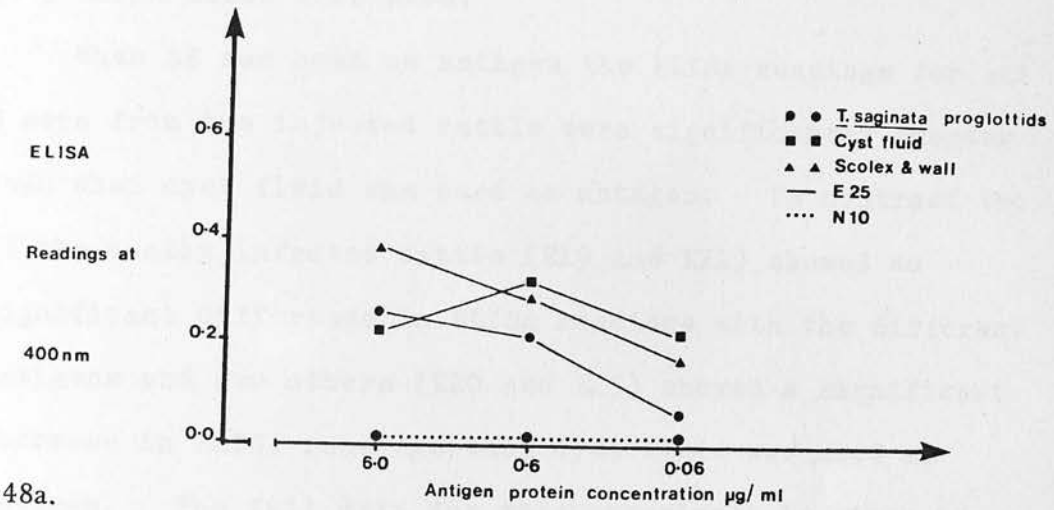


Figure 48a.

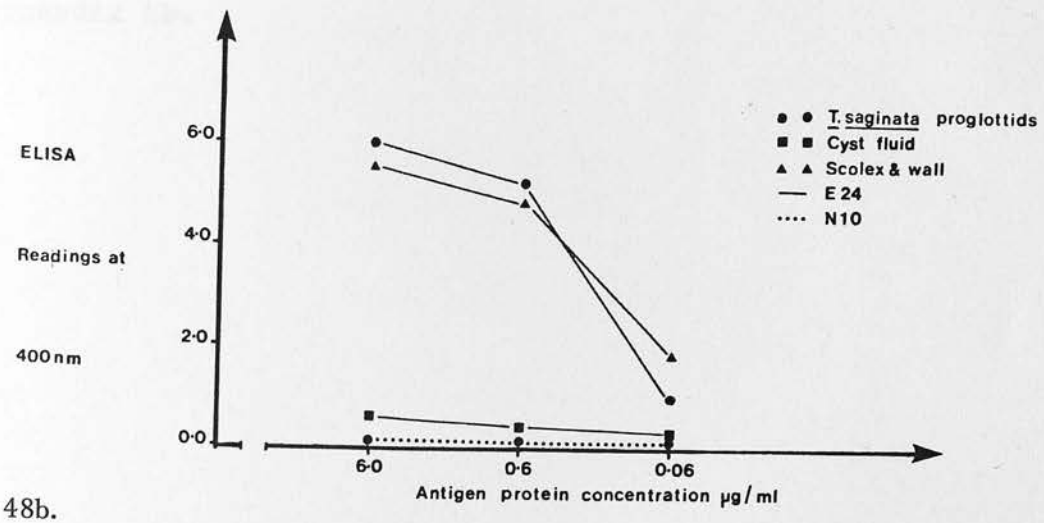


Figure 48b.

Figure 48. Antigen titration of T. saginata larval antigens for use in the ELISA technique (i) using sera from an orally infected calf (Figure 48a) (ii) using sera from a calf given a series of i/m injections with SE (Figure 48b).

These results were, therefore, rechecked (Table 29) using positive control sera from orally infected calves (E19, E20, E21 & E25) and from injected calves (E22, E23 & E24). A normal serum control was included. In this case groups of 3 ELISA tubes were used.

When SE was used as antigen the ELISA readings for all 3 sera from the injected cattle were significantly greater than when cyst fluid was used as antigen. In contrast two of the orally infected cattle (E19 and E21) showed no significant difference in ELISA readings with the different antigens and two others (E20 and E25) showed a significant increase in ELISA readings when cyst fluid was used as antigen. The full data for this experiment is given in appendix 4b.

Table 29 *T. saginata* cyst fluid and SE used as antigens in the ELISA technique.

Serum	Mean ELISA reading* at 400 nm ± sd		Significance (p)
	SE	Cyst fluid	
E19	0.163 ± 0.010	0.167 ± 0.006	> 0.05
E20	0.096 ± 0.011	0.157 ± 0.021	< 0.001
E21	0.186 ± 0.021	0.207 ± 0.031	> 0.05
E25	0.163 ± 0.000	0.218 ± 0.025	< 0.01
E22	0.270 ± 0.021	0.063 ± 0.006	< 0.001
E23	0.243 ± 0.027	0.040 ± 0.000	< 0.001
E24	0.360 ± 0.025	0.032 ± 0.003	< 0.001
N10	0.013 ± 0.010	0.000 ± 0.000	> 0.05

* Corrected to 3 d.p.

3.4.1.1.2.1. Sephadex G200 fractionated extracts of SE for use as antigens in the ELISA technique.

Untreated SE gave higher ELISA readings than the Sephadex G200 reprocessed extracts, (Figure 49). A protein concentration of 6 µg/ml was considered suitable for coating the ELISA tubes for all 4 antigen preparations.

Fractions 2.S.1. and 2.S.2. gave similar ELISA readings to the untreated SE but fraction 2.S.3. gave much lower ELISA readings (Figure 50). The full data for these experiments is given in appendix 4c and appendix 4d.

Sera from experimental cattle and normal bovine sera were tested using SE and the Sephadex G200 reprocessed extracts as antigens. Sets of 2 ELISA tubes were used and the results expressed as the ELISA readings minus the mean saline control reading (Table 30). Untreated SE gave higher ELISA readings than the fractionated extracts. There was little difference between the ELISA readings obtained with fractions S.1.1. and S.2.2., but fraction S.2.2. gave slightly higher ELISA readings.

Figure 49a.

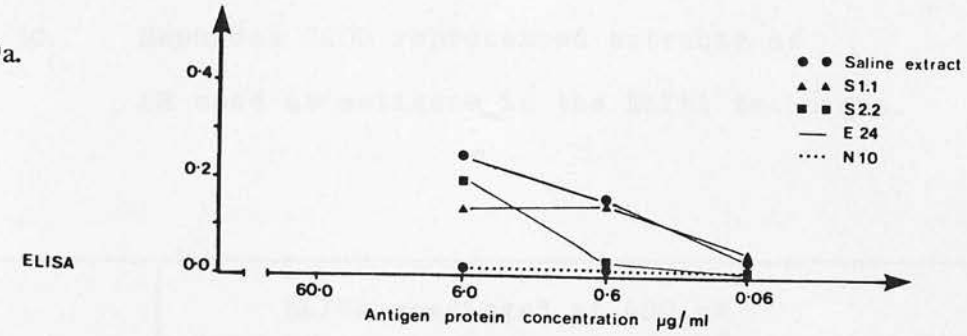


Figure 49b.

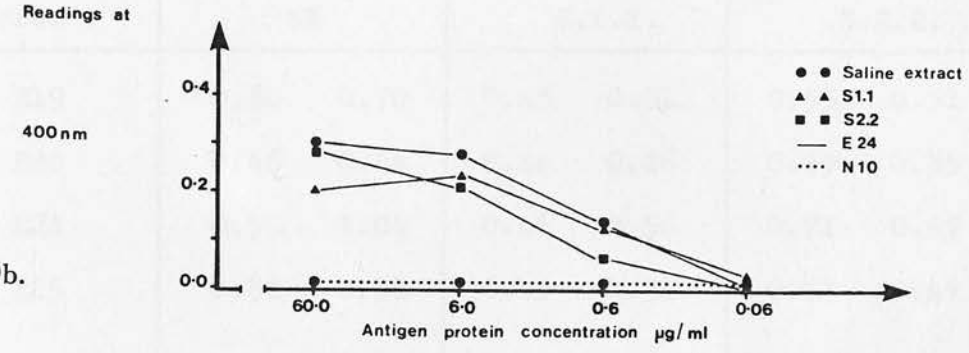


Figure 49a & b. Antigen titration of Sephadex G200 reprocessed extracts of SE used in the ELISA technique.

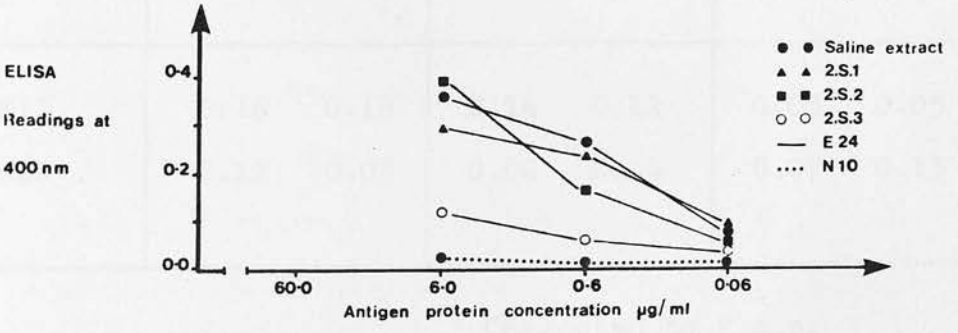


Figure 50. Antigen titration of Sephadex G200 fractions of SE used in the ELISA technique.

Table 30 Sephadex G200 reprocessed extracts of SE used as antigens in the ELISA technique.

Serum	ELISA readings* at 400 nm					
	SE		S.1.1.		S.2.2.	
E19	0.80	0.70	0.45	0.31	0.55	0.51
E20	0.46	0.44	0.24	0.26	0.29	0.35
E21	0.94	1.04	0.46	0.56	0.71	0.69
E25	0.80	0.68	0.45	0.31	0.63	0.49
E22	1.14	1.14	0.59	0.64	0.93	0.89
E23	0.76	0.90	0.56	0.61	0.73	0.77
E24	1.34	1.38	0.98	0.96	0.95	1.05
N11	0.16	0.18	0.14	0.12	0.05	0.05
N10	0.12	0.08	0.04	0.04	0.07	0.13

* Corrected to 2 d.p.

3.4.1.1.2.2. Absorption of a sample of serum from a T. saginata infected calf using Sephadex G200 fraction S.1.1.

A sample of serum (E21) was absorbed with an amount of Sephadex G200 fraction S.1.1. sufficient to remove all the HI activity. The absorbed serum was tested for activity in the ELISA technique using ELISA tubes coated with either untreated SE or fractions S.1.1. and S.2.2. (Table 31). Groups of 3 ELISA tubes were used and the results were compared with unabsorbed serum to obtain the percentage inhibition due to absorption. This percentage was calculated:-

$$\frac{R - RA}{R} \times 100$$

where R = the non absorbed serum reading

RA = the absorbed serum reading.

The mean and standard deviation of the percentage inhibition was then calculated for each group of tubes. The full data for this experiment is given in appendix 4e. Analysis showed that the ELISA readings obtained with S.1.1. coated ELISA tubes were more inhibited ($p < 0.01$) than those from tubes coated with either the crude saline extract ($p < 0.01$) or fraction S.2.2. ($p < 0.01$). There was no significant difference ($p > 0.05$) between the percentage inhibition of the serum when tested with the crude saline extract or fraction S.2.2. coated tubes.

Table 31 Percentage inhibition obtained in ELISA readings when a sample of serum from a T. saginata infected calf was absorbed with sufficient fraction S.1.1. to remove all the haemagglutinin activity from the serum.

Antigen used to coat ELISA tubes	Crude extract	S.1.1.	S.2.2.
Percentage inhibition of ELISA readings at 400nm ($\bar{x} \pm$ sd)	60.1 $\pm$ 1.99	66.8 $\pm$ 0.00	53.2 $\pm$ 4.68

3.4.1.2. Sephadex G200 fractionated extracts of SE used in the IDH technique.

Haemagglutination could not be detected when Sephadex G200 fractions 2.S.2. and 2.S.3. (3.3.2.2.1.3.) were used as coating antigens on the red blood cells in the IDH technique (Figure 51). The untreated SE and Sephadex G200 fraction 2.S.1. both gave positive results with the positive control serum (E21). For similar protein concentrations Sephadex G200 fraction 2.S.1. gave higher titres than SE. Purification of antigens by Sephadex G200 chromatography may have increased the sensitivity of the IDH technique.

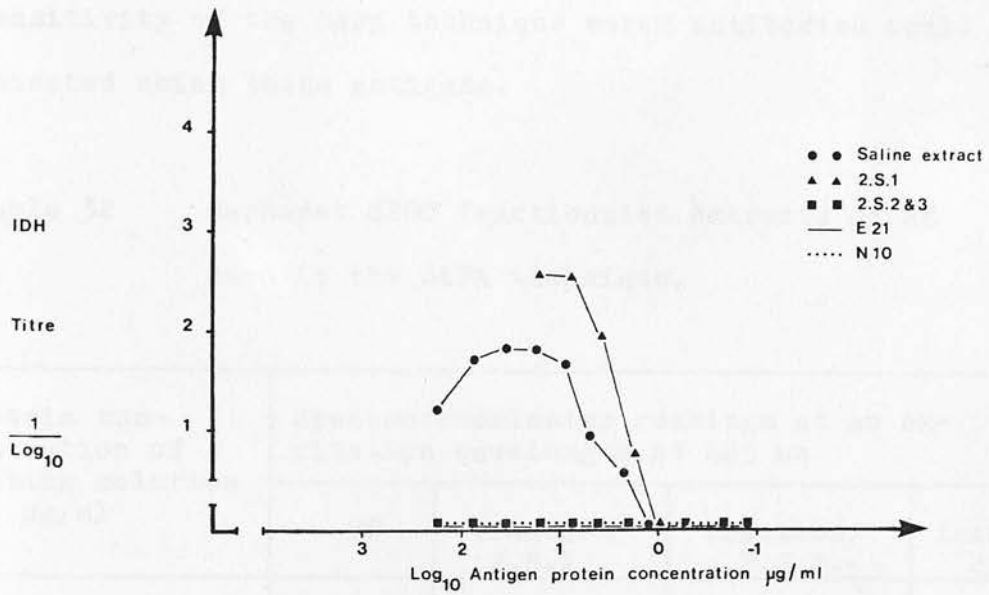


Figure 51. A comparison of untreated SE and Sephadex G200 extracts of SE used in the IDH technique.

3.4.1.3. Sephadex G200 fractionated extracts of SE used in the SAFA technique.

Sephadex G200 fractionated extracts of SE were used as antigens in the SAFA technique. Despite the lack of sensitivity of the SAFA technique serum antibodies could be detected using these antigens.

Table 32 Sephadex G200 fractionated extracts of SE used in the SAFA technique.

Protein concentration of coating solution µg/ml	Spectrofluorimeter readings at an excitation wavelength of 465 nm			
	SE	fraction 2.S.1.	fraction 2.S.2.	fraction 2.S.3.
25	0.1	0.14	0.06	0.04
12.5	0.1	0.11	0.08	0.05
6.75	0.11	0.09	0.03	0.02
3.875	0.1	0.06	0.03	0.01
1.9375	0.09	0.06	0.04	0.03

The results are expressed as the positive serum (E24) reading for each SAFA disc minus the corresponding normal serum (N10) reading. Both sera were used at a dilution of 1:2.5. The activity in fractions 2.S.2. and 2.S.3. may be a tailing effect from fraction 1. This could only be proved by absorption but the system was probably too insensitive to conduct such a study.

3.4.1.4. The micro gel precipitation technique.

The precipitin lines produced by this technique with the T. saginata system in cattle were very often faint, possibly because the antibody response to infection was so weak. Consequently, it was difficult to obtain good photographs of the gel precipitin lines (Figure 52).
The gel precipitin antigens, when detected in an antigen sample, were coded according to their positions relative to the serum well (Figure 53). At least 4 gel precipitin antigens were present in untreated SE but there were probably more. Gel precipitin antigen 1 may have been composed of 2 separate antigens. Generally serum E21 was used to test antigen fractions for gel precipitin activity as this serum had more precipitin antibodies than the other standard positive sera.

3.4.2. Serological response of cattle to experimental oral infection with T. saginata eggs.

3.4.2.1.1. Calves aged between 3 months and 1 year.

The serological responses of six cattle orally infected with T. saginata eggs at various ages (E1, E2, E3, E6, E7 and E8), were monitored by the IDH and ELISA techniques (Figure 54), using SE as antigen. The ELISA results are expressed as a percentage of a standard positive serum (E24). The IDH results are not so expressed because this would misleadingly suggest that the IDH technique was insensitive as

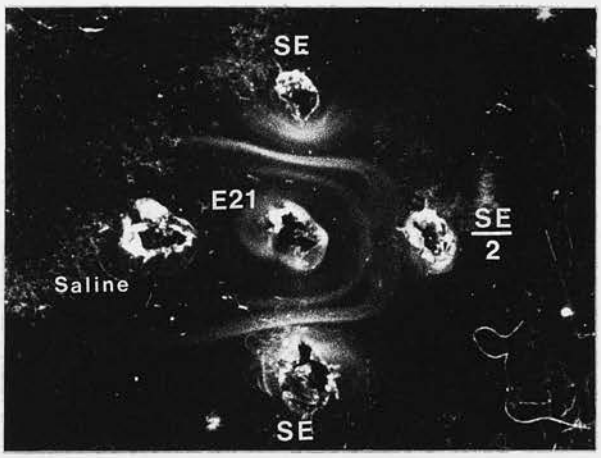


Figure 52. Gel precipitin antigens present in SE.

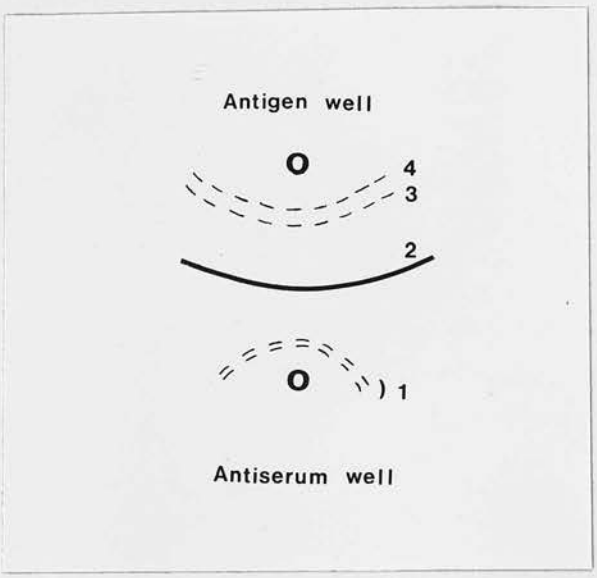


Figure 53. The code numbers given to the gel precipitin antigens present in SE.

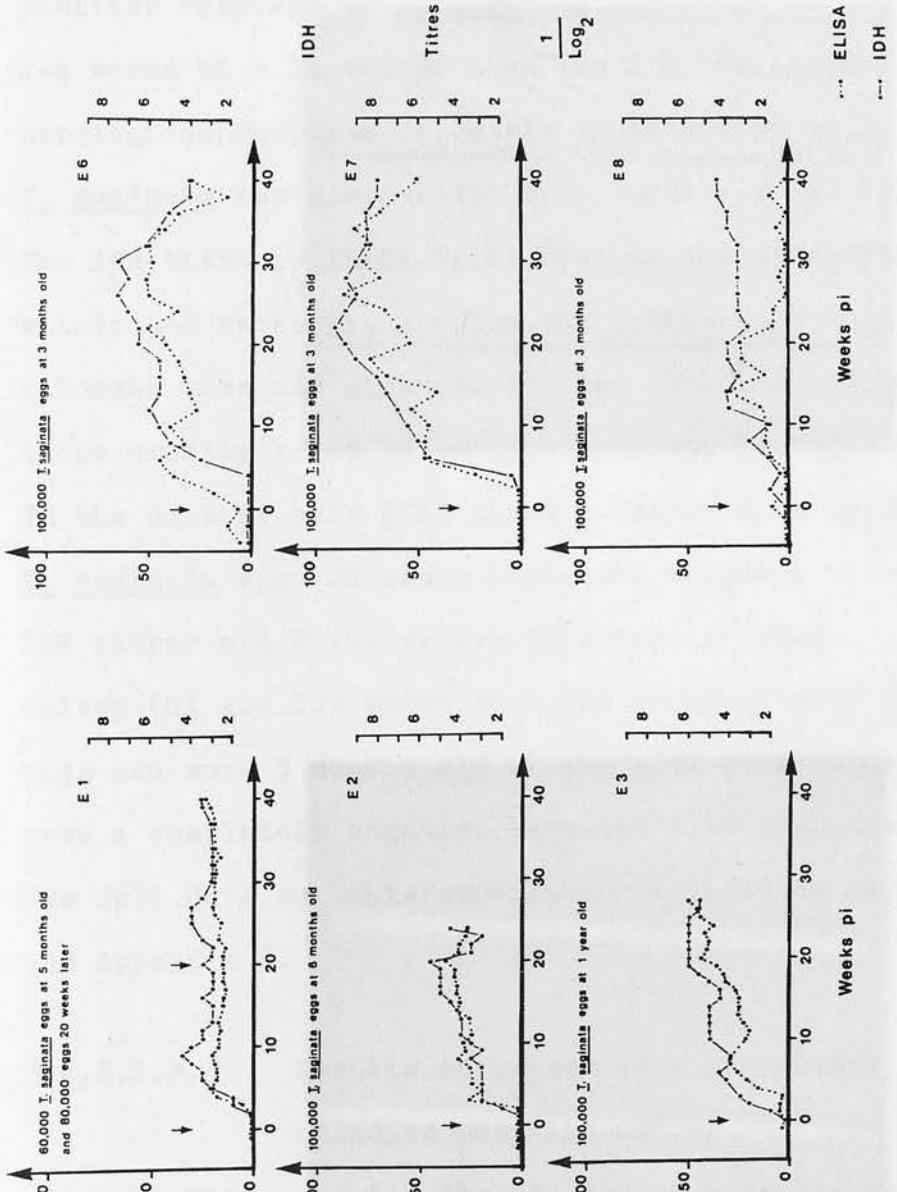


Figure 54. The serological response of calves to experimental oral infection with T. saginata eggs.

compared to ELISA when titres, as in this case are low.

The ELISA technique as conducted here detected a positive response to T. saginata infection in cattle by 2-4 weeks pi - no sooner than the IDH technique. The serological response of cattle to infection with T. saginata was similar for both ELISA and the IDH technique. The IDH titre or ELISA value rose to a sloping plateaux within 4-6 weeks pi. When the cattle were monitored for a longer time and given no further oral infections the titre usually began to decline by about the 30th week pi. In the case of calf (E1) given a repeat oral infection of T. saginata eggs 20 weeks after the original infection the IDH titres and ELISA values continued to rise. Two control calves (N1 and N2) which were not infected with T. saginata eggs and were 3 months old at the beginning of the experiment gave a completely negative response with both techniques. The full data for this experiment is given in appendix 5 and appendix 6.

3.4.2.1.2. Results expressed as a percentage of a standard positive serum.

The ELISA results clearly showed a marked response several weeks pi earlier than the IDH results (Figure 55). Titres for the IDH technique and readings for the ELISA technique were expressed as a percentage of a standard positive serum (E24). The ELISA values reached a sloping plateau within 8 weeks pi whereas the IDH results

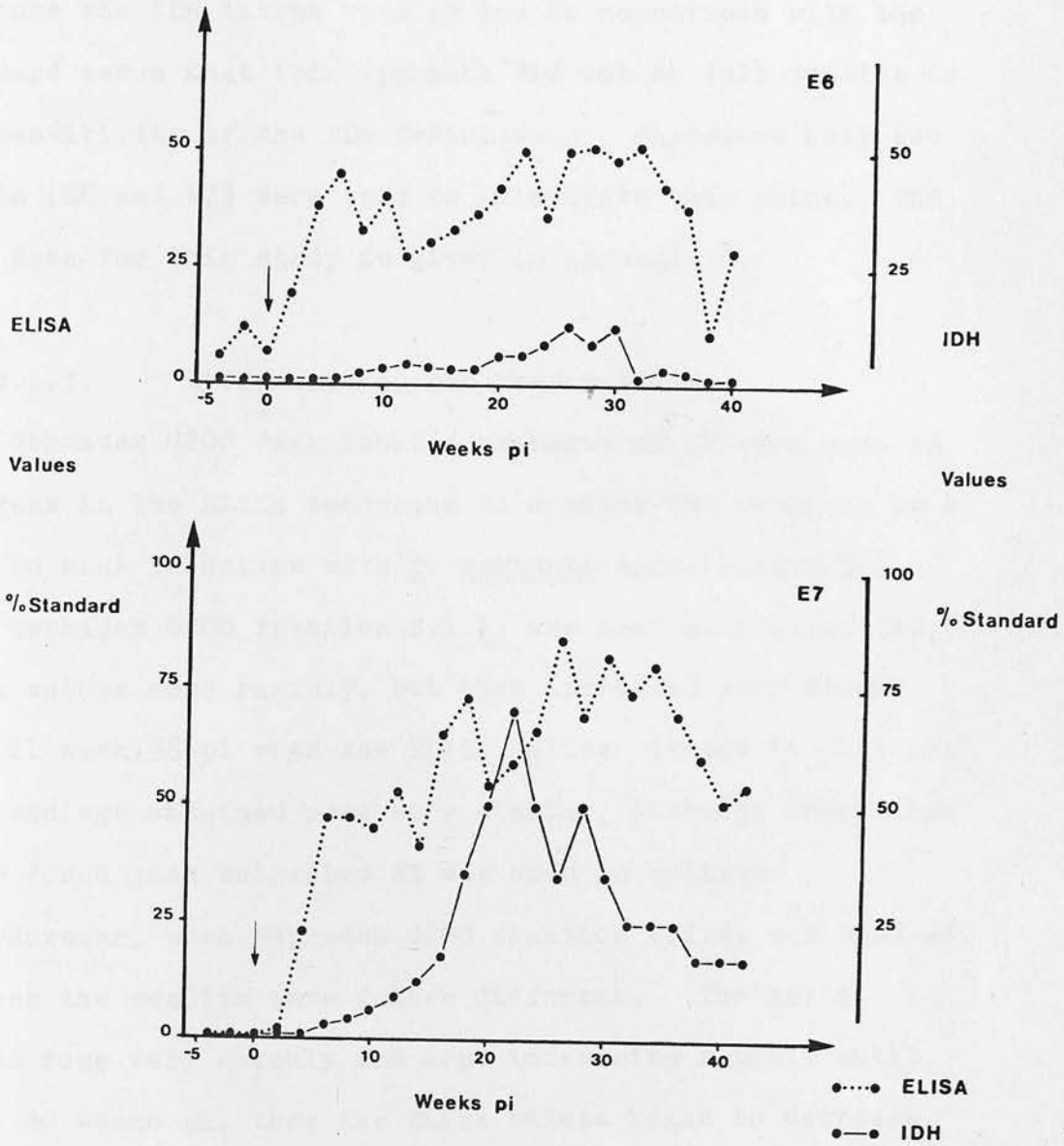


Figure 55. The serological response of two calves to experimental oral infection with T. saginata eggs with the results for both the IDH and ELISA techniques expressed as a percentage of a standard positive serum (E24).

indicated that over 16 weeks are required to reach maximal IDH values. However, in cattle with a poor serum antibody response the IDH titres were so low in comparison with the standard serum that this approach did not do full justice to the sensitivity of the IDH technique. Therefore only two calves (E6 and E7) were used to illustrate this point. The full data for this study is given in appendix 6.

3.4.2.1.3. Different antigen preparations.

Sephadex G200 fractionated extracts of SE were used as antigens in the ELISA technique to monitor the response of a calf to oral infection with T. saginata eggs (Figure 56). When Sephadex G200 fraction S.1.1. was used as antigen the ELISA values rose rapidly, but then increased very slowly up till week 36 pi when the ELISA values started to decline. The readings obtained were very similar, although lower than those found when untreated SE was used as antigen.

However, when Sephadex G200 fraction S.2.2. was used as antigen the results were rather different. The ELISA values rose very quickly and kept increasing rapidly until about 20 weeks pi, then the ELISA values began to decrease. The results are expressed as a percentage of a standard positive serum (E25) and the full data is given in appendix 7.

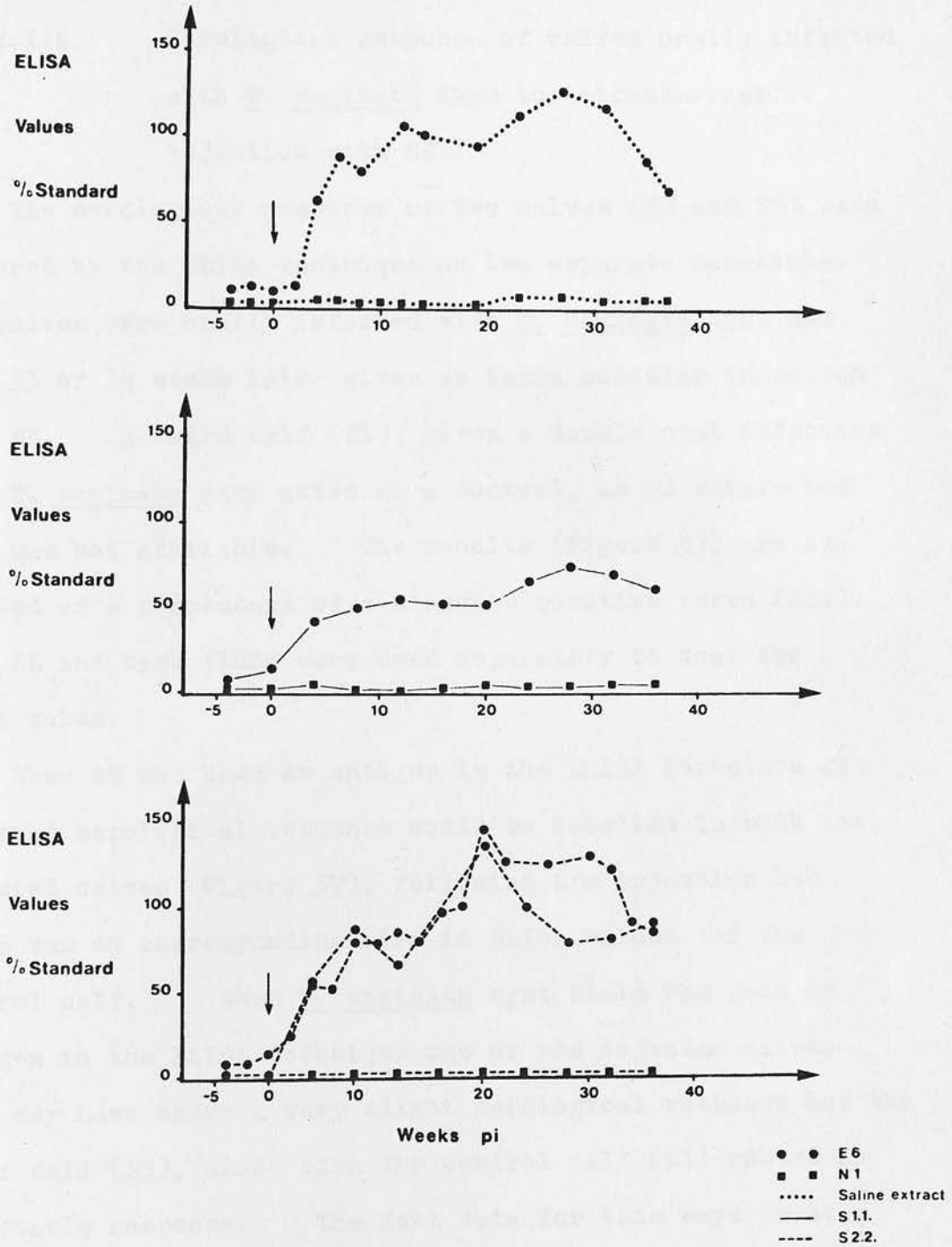


Figure 56. The serological response, as monitored by the ELISA technique, of a calf orally infected with T. saginata eggs when Sephadex G200 fractionated extracts of SE were used as antigens.

3.4.2.1.4. Serological response of calves orally infected with T. saginata eggs to intramuscular injection with SE.

The serological response of two calves (E2 and E3) were measured by the ELISA technique on two separate occasions. The calves were orally infected with T. saginata eggs and then 33 or 34 weeks later given an intra muscular injection with SE. A third calf (E1), given a double oral infection with T. saginata eggs acted as a control, as an uninfected calf was not available. The results (Figure 57) are expressed as a percentage of a standard positive serum (E24). Both SE and cyst fluid were used separately to coat the ELISA tubes.

When SE was used as antigen in the ELISA technique an enhanced serological response could be detected in both the injected calves (Figure 57), following i/m injection but there was no corresponding rise in ELISA values for the control calf. When T. saginata cyst fluid was used as antigen in the ELISA technique one of the injected calves (E2) may have shown a very slight serological response but the other calf (E3), along with the control calf (E1) showed no detectable response. The full data for this experiment is given in appendix 8.

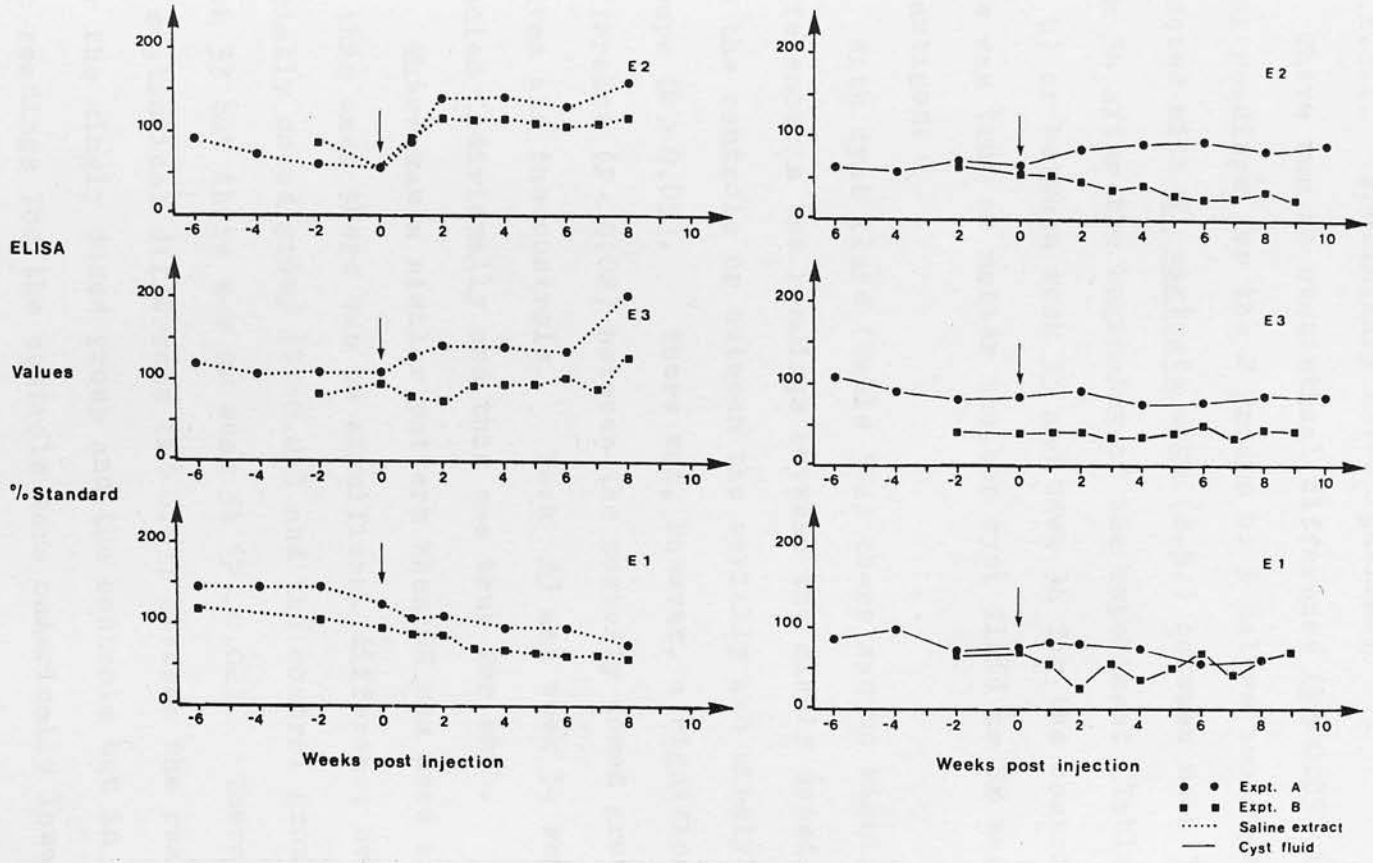


Figure 57. The serological response of calves orally infected with T. saginata eggs to an i/m injection of SE. Either T. saginata cyst fluid or SE was used as test antigen.

3.4.2.2. The serological response of calves to neonatal oral infection with T. saginata eggs.

3.4.2.2.1. Preliminary investigations.

There was no statistical difference ($p > 0.05$) in the ELISA readings for the 2 groups of 5 calves neonatally infected with T. saginata eggs (2.5.) between week 33 and week 34 after the beginning of the experiment (Tables 33a and b) or between week 33 and week 34 for the control group. This was true no matter whether cyst fluid or SE was used as antigen.

With cyst fluid (Table 33a) there was no significant difference in the readings between the singly dosed group and the controls or between the serially and singly dosed groups ($P > 0.05$). There was, however, a significant difference ($P < 0.02$) between the serially dosed group of calves and the controls. Week 33 and week 34 were studied individually and this was true for both.

There was a similar pattern when SE was used as antigen. In this case there was no significant difference between the serially dosed group ($P > 0.05$) and the control group on week 33 but there was on week 34 ($P < 0.02$). There was no statistical difference ($P > 0.05$) between the readings for the singly dosed group and the controls but in all cases the readings for the controls were numerically lower. The full data for this experiment is given in appendix 9.

Table 33a ELISA readings for two groups of neonatally infected calves and a control group with *T. saginata* cyst fluid used as antigen.

Cyst fluid antigen	ELISA readings* at 400 nm		
Weeks pi	Serial group $\bar{x} \pm$ sd	Single group $\bar{x} \pm$ sd	Controls $\bar{x} \pm$ sd
33	0.048 ± 0.029	0.026 ± 0.020	0.006 ± 0.006
34	0.055 ± 0.032	0.033 ± 0.031	0.008 ± 0.008

* Corrected to 3 d.p.

Table 33b ELISA readings for two groups of neonatally infected calves and a control group with SE used as antigen.

SE	ELISA readings* at 400 nm		
Weeks pi	Serial group $\bar{x}$ + sd	Single group $\bar{x}$ + sd	Controls $\bar{x}$ + sd
33	0.067 ± 0.044	0.048 ± 0.024	0.025 ± 0.005
34	0.064 ± 0.027	0.048 ± 0.031	0.026 ± 0.004

* Corrected to 3 d.p.

3.4.2.2.2. Serological response over a 14 month period post infection.

In view of the low readings already obtained (3.2.2.2.1.) the sera were diluted to 1:25 and not 1:50 as usual. SE was used as the antigen to coat the ELISA tubes. The ELISA readings were taken with a 1 cm light path cuvette on a Pye Unicam SP400 spectrophotometer and the complete data for the experiment is given in appendix 10.

The control calves gave relatively high readings in the first four months of the experiment after which the readings levelled off. The calves in the two experimental groups also gave high readings at the beginning of the experiment (Figure 58). In certain cases the ELISA values rose considerably in the first month pi dropping down again in the second to third months pi.

In the serially dosed group one calf (E12) did not respond to infection at all whereas another (E11) did not respond in the first month but later did give quite high ELISA values. The other calves all responded initially and thereafter gave varying ELISA values.

With the singly dosed group, two calves (E15 and E18) did not display a strong serological response, although E18 gave increased ELISA values in the first month. The other three calves all gave increased ELISA values in the first month but one (E14), gave a very low response thereafter. Another (E16) did not respond well until 9 - 12 months pi but the third (E17) gave a very pronounced serological response

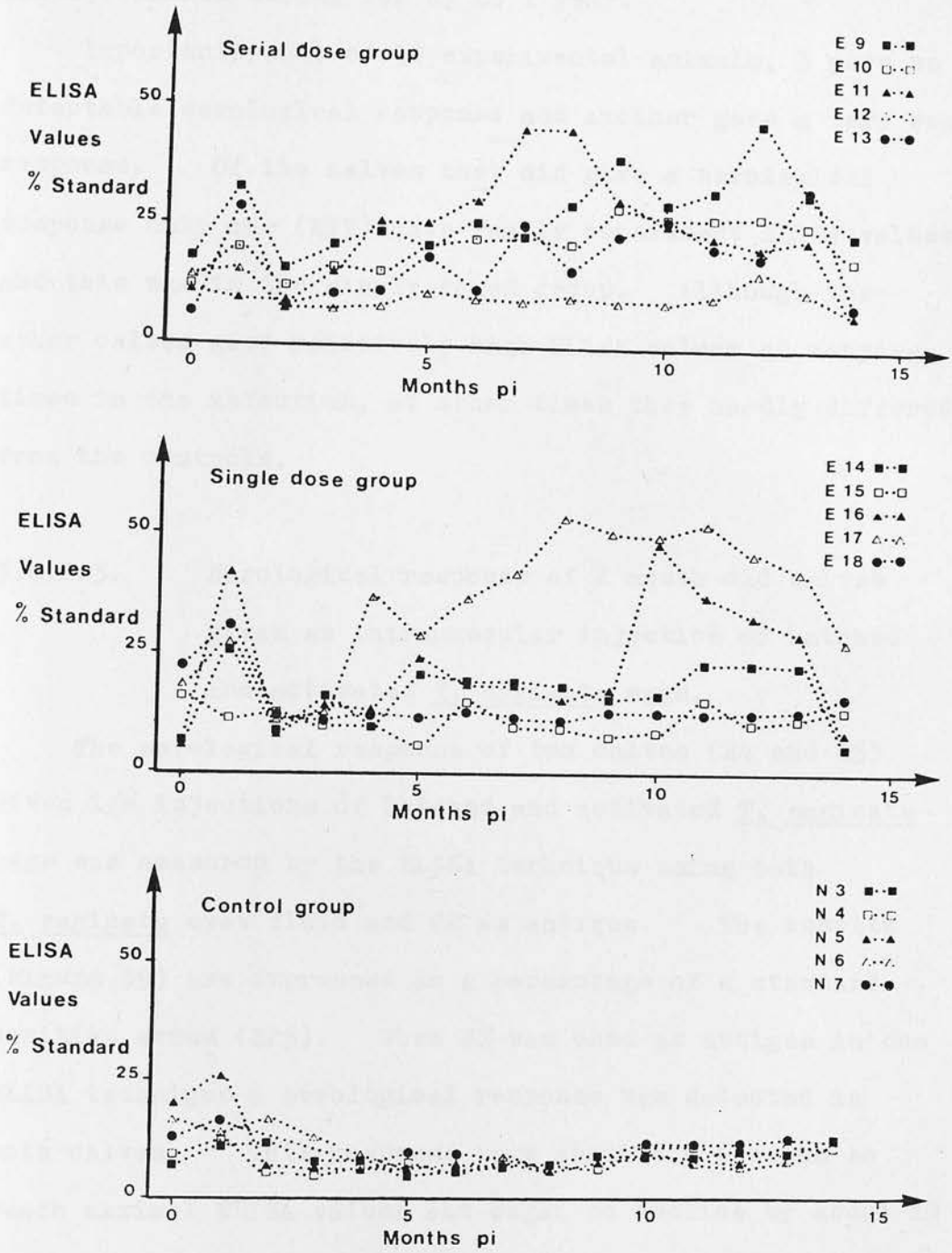


Figure 58. The serological response of neonate calves to oral infection with T. saginata eggs.

which remained strong for up to 1 year.

Importantly, out of 10 experimental animals, 3 gave no detectable serological response and another gave a very weak response. Of the calves that did give a serological response only one (E17) gave really consistant ELISA values and this was in the singly dosed group. Although the other calves gave relatively high ELISA values at varying times in the infection, at other times they hardly differed from the controls.

3.4.2.3. Serological response of 2 month old calves given an intramuscular injection of hatched and activated T. saginata eggs.

The serological response of two calves (E4 and E5) given i/m injections of hatched and activated T. saginata eggs was measured by the ELISA technique using both T. saginata cyst fluid and SE as antigen. The results (Figure 59) are expressed as a percentage of a standard positive serum (E25). When SE was used as antigen in the ELISA technique a serological response was detected in both calves. This response took about 5 - 6 weeks to reach maximal ELISA values and began to decline by about 10 weeks after infection. There was not such a marked serological response detected when T. saginata cyst fluid was used as antigen. One calf (E4) hardly responded at all and calf (E5) only gave a slight response. The full data for this experiment is given in appendix 11.

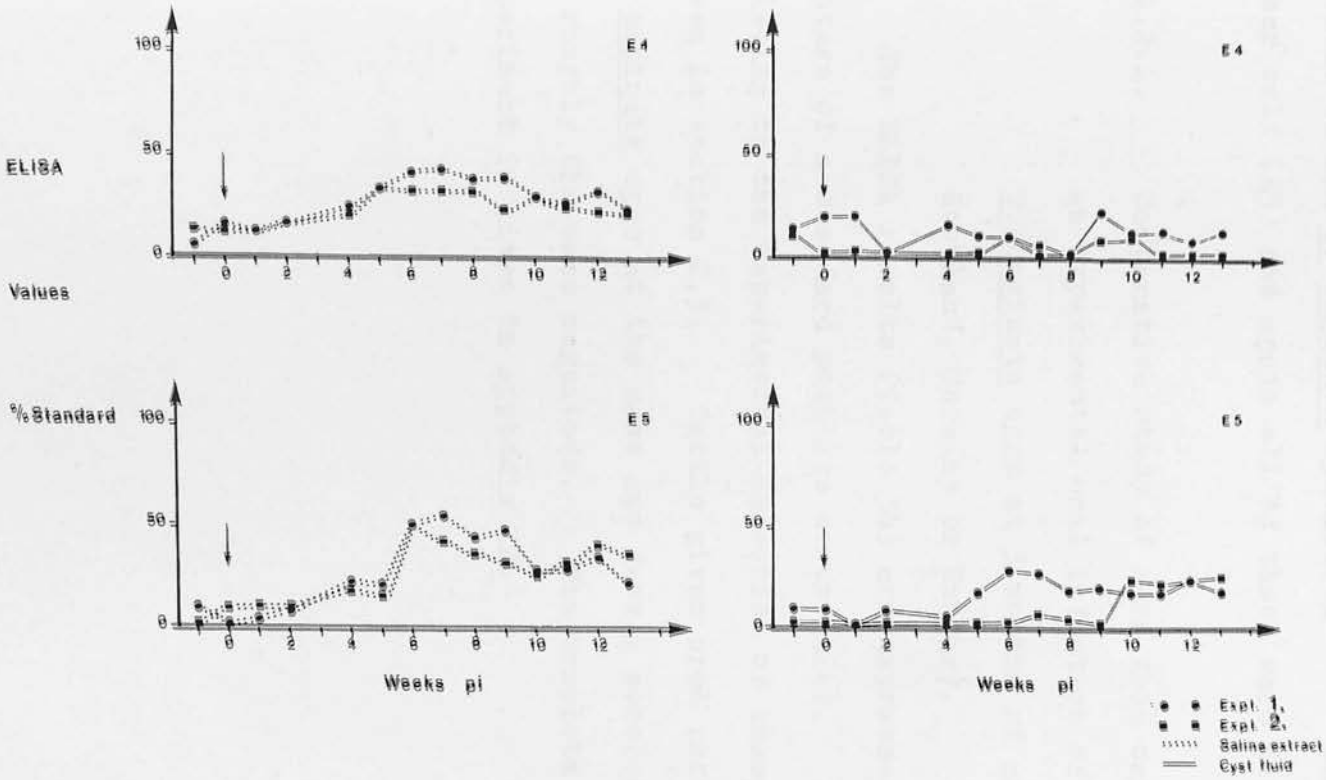


Figure 59. The serological response of two calves given i/m injections of hatched and activated T. saginata oncospheres. Either T. saginata cyst fluid or SE was used as antigen.

When these calves were slaughtered only 9 T. saginata cysticerci were found in calf (E4), all of which were alive. A total of 171 T. saginata cysticerci were found in the other calf (E5) and again all of these were alive.

3.4.2.4. Comparative study of sera from cattle given an experimental oral infection of 100,000 T. saginata eggs at 3 months of age in Scotland, Germany or Hungary.

The ELISA results (Table 34) are expressed as a percentage of a standard positive serum (E24). The details relating to the experimental infection of these calves are given in section 2.5. Cattle given oral infections with T. saginata eggs at the same age gave a serological response of roughly the same magnitude. The complete data for this experiment is given in appendix 12.

Table 34 Comparative study of sera from experimentally infected cattle* from Scotland, Germany and Hungary.

Weeks post infection	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	42	60
Scottish calves E6	12		7		19		38		45		33		40		27		30		33	48	
E7	0		0		2		23		47		47		45		53		41		65	54	
E8	0		9		0		0		9		12		11		14		29		12	6	
German calves E26		9				8												64			33
E27		2				13												51			
E28															44		.				
E29																		44			
Hungarian calves E30										48	31	39					34				
E31										23	30	32					29				

* Orally dosed with 100,000 T. saginata eggs at 3 months of age.

3.4.3. Serological work with cattle from the field.

3.4.3.1. Serological investigation on serum samples taken from cattle on three different farms.

The following summarises the results obtained from the herd pictures of a serological study on calves from three Scottish farms (farms A, B and C) the details for which are given in section 2.5. The full data for these results is given in appendix 13.

3.4.3.1.1. Serum protein concentration.

The protein concentration in the serum samples was estimated by the biuret reaction and the mean and standard deviations for each farm calculated (Table 35). There was no significant difference ($p > 0.05$) in the mean protein content of the sera from cattle on farm A and farm B, but there was significantly more protein ($p < 0.001$ and $p < 0.05$) in the sera from cattle on farm C compared with farms A and B.

Table 35 Mean serum protein content* for cattle on farms A, B and C.

Farm	$\bar{x}$ Protein concentration (g/100 ml)	Standard deviation	Sample size
A (infected)	6.190	$\pm$ 0.339	24
B (control)	6.464	$\pm$ 0.573	14
C (control)	6.945	$\pm$ 0.383	11

All subsequent tables marked * are corrected to 3 d.p. ** to 2 d.p. and *** to 4 d.p.

3.4.3.1.2. Serum globulin concentration.

The percentage concentration of globulin in the serum samples was estimated by electrophoresis (2.7.11.) and the globulin concentration calculated from the total protein concentration of the samples as estimated by the biuret reaction.

Table 36 Mean globulin concentration* for serum samples from cattle on farms A, B and C.

Farm	$\bar{x}$ Globulin conc. g/100 ml	Standard deviation	Sample size
A (infected)	3.489	$\pm$ 0.394	24
B (control)	3.266	$\pm$ 0.434	14
C (control)	4.218	$\pm$ 0.252	11

There was no statistical difference ($p > 0.05$) between the globulin concentration in the sera of cattle on farms A and B (Table 36), but there was a significantly lower globulin concentration ($p < 0.001$) in the sera from cattle on both farms A and B than from cattle on farm C.

3.4.3.1.3. Serum albumin concentration.

The percentage albumin concentration in the serum samples was estimated by electrophoresis (2.7.11.) and the albumin concentration was calculated from the total protein concentration of the serum samples as estimated by the buiret reaction.

Table 37 Mean albumin concentration* ofserum samples from cattle on farms A, B and C.

Farm	$\bar{x}$ Albumin concentration g/100 ml	Standard deviation	Sample size
A (infected)	2.693	$\pm$ 0.276	24
B (control)	3.198	$\pm$ 0.271	14
C (control)	2.728	$\pm$ 0.277	11

There was significantly less ($p < 0.001$) albumin in the sera from farm A than farm B, but signficantly more ($p < 0.001$) albumin in the sera from farm B than farm C. However, there was no significant difference ($p > 0.05$) between the albumin concentration in sera from farms A and C (Table 37).

3.4.3.1.4. The indirect haemagglutination (IDH) technique.

Low IDH titres were obtained with the sera from farms A, B and C. The titres were, therefore, taken as the last dilution of serum which showed any signs of haemagglutination and the results expressed as $\frac{1}{\text{Log}_{10}}$ titre (Table 38). There was no significant difference ($p > 0.05$) found in the mean IDH titres of the sera from cattle on farms A and B. Farm C, however, had a significantly lower mean titre than either farms A or B ($p < 0.01$ and $p < 0.001$ respectively).

***

Table 38 The mean IDH titres for sera from cattle on farms A, B and C.

Farm	Mean IDH titre $\frac{1}{(Log_{10})}$	Standard deviation	Sample size
A (infected)	0.8926	± 0.5225	24
B (control)	1.0352	± 0.4147	14
C (control)	0.2737	± 0.3411	11

3.4.3.1.5. The micro gel precipitation (MGP) technique.

With the exception of one calf on farm A all the sera gave negative results with the micro-gel precipitation technique.

3.4.3.1.6. The soluble antigen fluorescent antibody (SAFA) technique.

The sera from the three farms were tested by the SAFA technique. The results (Table 39) are expressed as a percentage of a standard positive serum (E24).

Table 39 SAFA results** from sera of cattle on farms A, B and C.

Farm	$\bar{x}$ SAFA value	Standard deviation	Sample size
A (infected)	22.27	± 16.10	24
B (control)	19.54	± 10.67	14
C (control)	12.50	± 11.18	11
B+C pooled control	16.44	± 11.25	25

There was no significant difference ($p > 0.05$) in the SAFA values between any of these farms. However farm C did give lower readings than either farms A or B.

3.4.3.1.7. The enzyme linked immunosorbent assay (ELISA).

The sera from the three farms were tested using the ELISA technique. In addition to SE, Sephadex G200 fractionated extracts of SE were also used as antigen. These were the reprocessed fractions S.1.1. and S.2.2. The results are expressed as a percentage of a standard positive serum (E24.).

Table 40 ELISA results* for farm A.

Antigen used for coating ELISA tubes	Mean ELISA value	Standard deviation	Sample size
SE	13.575	± 10.619	24
S.1.1.	25.521	± 11.802	24
S.2.2.	28.133	± 14.946	24

The ELISA values for farm A using the untreated SE were significantly lower ($p < 0.001$) than both antigens S.1.1. and S.2.2. (Table 40). However, there was no significant difference ($p > 0.05$) between the readings obtained for fractionated antigens S.1.1. and S.2.2.

In a similar experiment on the sera from farm B (Table 41) there was no significant difference ($p > 0.05$) in the readings obtained from the three different antigens used, possibly because of the large variation in the ELISA values. When the results for each individual serum were inspected (appendix 13) it was found that 2 of the sera gave excessively high readings. If these outliers were omitted from the calculations (Table 42), the readings for the untreated SE were significantly lower ($p < 0.02$) than the readings for antigen S.2.2. but there was no significant difference ($p > 0.05$) between the readings for untreated SE and fraction S.1.1.

Table 41 ELISA results* for farm B (outliers included).

Antigen used to coat ELISA tubes	Mean ELISA values	Standard deviation	Sample size
SE	16.380	± 20.978	14
S.1.1.	17.143	± 24.738	14
S.2.2.	28.929	± 34.715	14

Table 42 ELISA results* for farm B (outliers excluded).

Antigen used to coat ELISA tubes	Mean ELISA values	Standard deviation	Sample size
SE	8.875	± 3.156	12
S.1.1.	9.000	± 2.593	12
S.2.2.	16.833	± 9.523	12

The results obtained from farm C (Table 43) were very similar to those obtained with farm B when the two outliers were omitted. Again there was no significant difference ($p > 0.05$) between the ELISA values obtained with untreated SE and S.1.1. but the untreated SE gave a significantly lower ($p < 0.02$) reading than antigen S.2.2. as did antigen S.1.1. ($p < 0.01$).

Table 43 ELISA results* for farm C.

Antigen used to coat ELISA tubes	Mean ELISA values	Standard deviation	Sample size
SE	9.891	± 3.652	11
S.1.1.	8.182	± 4.276	11
S.2.2.	22.000	± 14.540	11

For the purposes of analysis the results from control farm B and C were pooled, both with the two outliers on farm B included (Table 44) and with them omitted (Table 45).

The ELISA values for the three farms for each of the antigen preparations were compared, first with the two outliers in farm B included and then with them removed. As with the SAFA technique with the untreated SE there was no significant difference ($p > 0.05$) in the ELISA results between any of the farms even when the two outliers on farm B were omitted.

When antigen S.2.2. was used to coat the ELISA tubes and the two outliers on farm B included there was no significant difference detected between any of the farms ($p > 0.05$).

Table 44 Pooled ELISA results* for farms B and C. (outliers included).

Antigen used to coat ELISA tubes	Mean ELISA values	Standard deviation	Sample size
SE	13.524	15.960	25
S.1.1.	13.200	18.966	25
S.2.2.	25.880	27.444	25.

Table 45 Pooled ELISA results* for farms B and C (outliers omitted).

Antigen used to coat ELISA tubes	Mean ELISA values	Standard deviation	Sample size
SE	9.361	3.363	23
S.1.1.	8.609	3.443	23
S.2.2.	19.300	12.182	23

However, when these outliers were omitted farm A gave statistically higher ELISA values ($p < 0.05$) than farm B and the pooled results from farms B and C ($p < 0.05$), but there was still no significant difference ($p > 0.05$) between the ELISA values for farm A and farm C or between the control farms B and C.

The results obtained with antigen S.l.l. were more interesting. When the outliens from farm B were included farm A gave significantly higher ($p < 0.001$) ELISA values than farm C and also ($p < 0.01$) than the pooled control farms B and C. However there was still no significant difference ($p > 0.05$) between farm A and the control farm B or between farm B and farm C. When the two outliens on farm B were excluded farm A gave significantly higher ELISA values ($p < 0.001$) than both farm B and farm C separately and when these results were pooled. However, there was no statistical difference ($p > 0.05$) between farm B and farm C, the two control farms.

3.4.3.2. Serological investigation into serum samples taken from cattle on a Dutch farm which had cattle naturally infected with T. saginata cysticerci.

The experimental observations on these 5 Dutch cattle did not fully agree with the slaughter information (2.5.). In December 1974, D1, D2, D4, and D5 were all considered to give a positive result with the IDH technique, whereas D3 gave a doubtful result (Dr. F. van Knapen, pers. comm.). By March to May 1975, when these serum samples were taken, the IDH titres had fallen (Figure 60) and the ELISA values were also low. On slaughter T. saginata cysticerci were found in D2, D3 and D4 but not in D1 or D5.

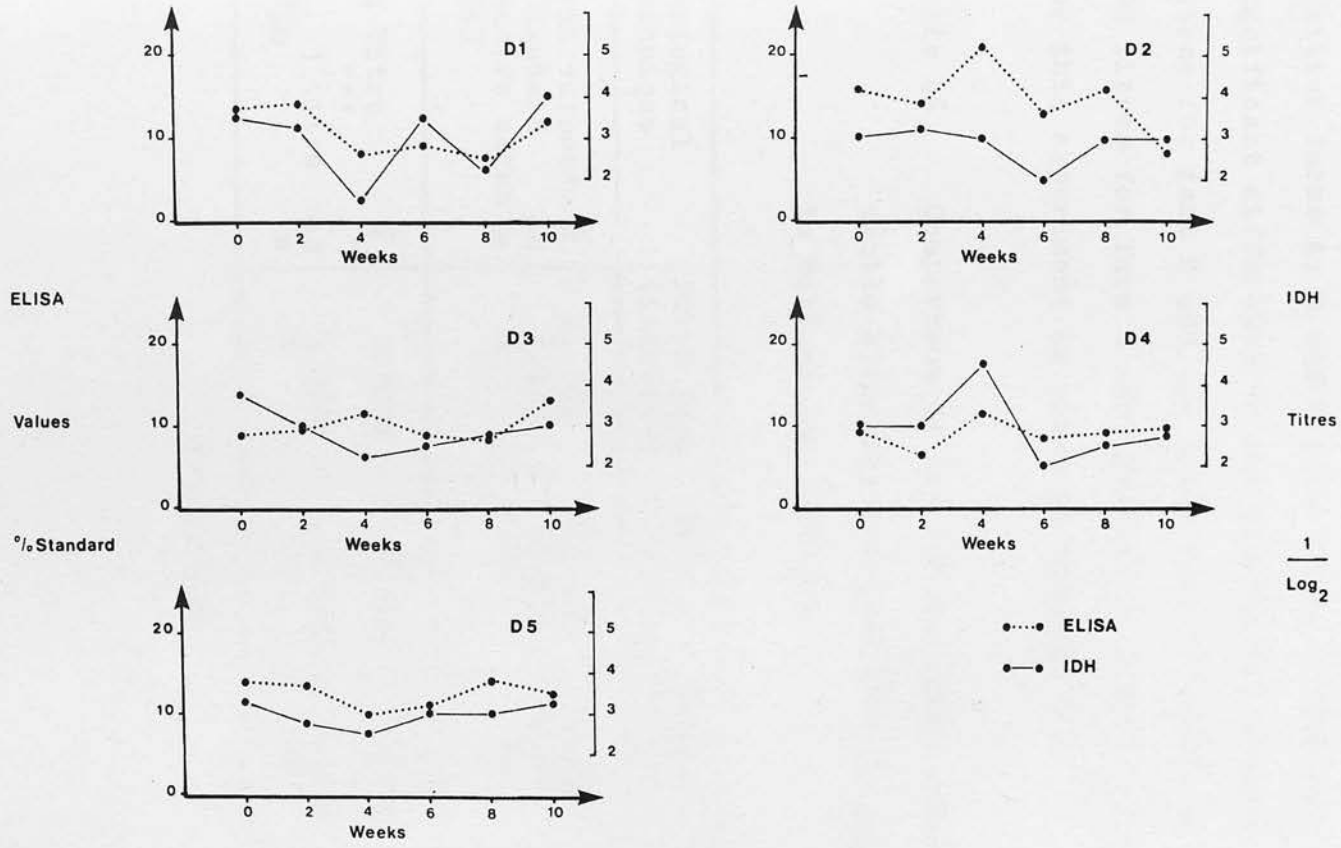


Figure 60. The serological response of five Dutch cattle naturally infected with T. saginata eggs. The response was measured over a ten week period.

In most cases there was no significant difference ($p > 0.05$) detected (Table 46) in either the IDH or ELISA values between these Dutch sera and the sera from the Scottish farms A, B and C (3.4.3.1.). The only significant difference in the results was between the IDH titres for farm C and the Dutch sera ($p < 0.01$) although the IDH titres for farm C were particularly low. The full data for this experiment is given in appendix 14.

Table 46 Comparison of the IDH and ELISA results from cattle naturally infected with T. saginata in Holland and Scotland.

Serological technique		Dutch Farm (infected)	Farm A (infected)	Farm B (control)	Farm C (control)
ELISA values* % Standard Positive serum (E24)	$\bar{x}$	11.500	13.575	16.380	9.891
	sd	2.130	10.619	20.978	3.652
	n	5	24	14	11
IDH Titre ($\frac{1}{Log_{10}}$)*** titre	$\bar{x}$	0.8679	0.8926	1.0352	0.2737**
	sd	0.0187	0.5225	0.4147	0.3411
	n	5	24	14	11

(** $p < 0.01$)

CHAPTER 4

4. Discussion.

4.1. Introduction.

There is at present a renewal of interest in the use of immunological techniques in various parasitic infections, mainly to detect serum antibody responses. Recently developed sensitive techniques such as ELISA and the defined antigen substrate spheres (DASS) system (Deelder & Streefkerk, 1975) are being used. These techniques use enzyme labelled antisera as the indicator system and result in a reaction product visible to the naked eye.

If techniques such as these are to be effective, pure and specific antigens are required, information is needed on their immuno-chemistry, their possible molecular structure and their relative antigenic activities. Fortunately techniques such as ELISA require only miniscule amounts of antigen, which is convenient because any purification technique usually results in a great loss of material.

These new serological techniques should be of value in monitoring the response to experimental T. saginata infections in cattle. In addition, perhaps in a modified form, they may be of use in detecting natural infections and possibly facilitate the development of protective vaccines. The detection of T. saginata cysticerci in cattle is only one of the areas which may benefit from development of these techniques.

4.2. Immunochemistry.

4.2.1. Preliminary work.

Globular proteins as antigens are generally heat labile whereas carbohydrates are generally heat stable at 100°C for 30 minutes or even more. The molecules responsible for HI activity in SE were at least partially resistant to heat treatment at 100°C for 30 minutes as was gel precipitin antigen 2 (3.3.1.1.), which suggested that the antigenically active components of these molecules may not be globular protein. Machnicka (1974) found that her polysaccaride fraction Calk had most activity in the IDH technique with untanned erythrocytes, although a protein fraction was most active in the IDH technique with tanned cells. Gel precipitin antigen 1 resisted heating at 65°C for 30 minutes, but gel precipitin antigens 3 and 4 were lost on heating at 56°C for 30 minutes, which suggested that these may be globular proteins. Machnicka (1974) found gel precipitin antigens associated with her protein and nucleoprotein fractions B_1, B_2, $N_{0.1}$ and also with carbohydrate fractions C, C_F and Calk from extracts of T. saginata proglottids.

The majority of the antigens responsible for HI activity and gel precipitin antigen 2 were precipitated by 0.25 M TCA (3.3.1.2.) as was most of the protein and carbohydrate. Similarly most of the HI antigens and gel precipitin antigen 2 were precipitated by 60 per cent saturated ammonium sulphate along with most of the protein and carbohydrate (3.3.1.3.). These results agreed with Sokolovskaya (1966), who found that the fraction of a T. saginata

cysticercal extract which was precipitated in 65 per cent saturated ammonium sulphate was active in the IDH technique.

4.2.2. Chromatography of SE.

In column chromatography, monitoring the column effluents at 220 nm gave a better indication of the presence of components of SE, especially when working with small quantities containing low concentrations of protein (3.3.2.2.1.2.). Monitoring column effluents at 254 nm mainly recorded the protein concentration or, more precisely, the tryptophane, tyrosine and phenylalanine content of the proteins. Many substances including carbohydrate and protein absorb light at 220 nm, which accounted for the improved recording at this wavelength. However, a survey of the literature showed that the most commonly used wavelengths for monitoring column effluents were 254 nm and 280 nm.

4.2.2.1. Gel filtration.

Of the gel filtration techniques used to fractionate SE (3.3.2.2.), Sephadex G200 gave the best results as it effectively separated the large molecules responsible for most of the HI activity from the smaller molecules containing the majority of the gel precipitin activity. Sepharose 6B and 4B not only tended to spread the HI activity through the fractions, as did Sephadex G200, but

also to spread the gel precipitin antigens, thus giving a poor separation. However, Sepharose 4B fractionation also removed some large molecules with little or no antigen activity (3.3.2.2.3.).

The peak for fraction 1.S4B.3. (Figure 34) was much larger than fraction 2.S4B.3. (Figure 35). This may have been due to molecular aggregation or complexing of the sample first applied to the S4B column. It was also noted that the total area under the trace was greater for the first Sepharose 4B fractionation than for the second, despite the fact that less material was applied to the column in the first case than in the second. This probably indicated that the gain control on the spectrophotometer (2.6.2.1.) had been altered between the first and second S4B chromatographic runs.

The antigens active in the HI technique were of a molecular weight equal to or greater than 10^6 if they were globular or 10^5 if they were linear. These molecules were mainly eluted in the first fraction pool on Sephadex G200 chromatography although there was a tailing effect throughout the trace (3.3.2.2.1.3.). This trailing effect may be partly inherent in the technique but may also be due to the presence of active fragments of the large HI antigens. Gel precipitin antigen 1 was also present in fraction pool S.1. The majority of the gel precipitin antigens were in the second Sephadex G200

fraction pool (S.2.), where precipitin antigens 2, 3 and 4 were present in greatest concentration. This fraction pool also contained some HI activity and corresponded to molecules that had a molecular weight of about 10^5 if they were globular or 3×10^4 if they were linear.

Concentrating SE, either by dialysis or by ultrafiltration with a membrane of molecular weight cut off of 1,000, removed a large amount of carbohydrate (3.3.2.2.1.3.). Fortunately the antigen activity appeared to be associated with large molecules which could not pass through visking tubing or the ultra filtration membrane.

On reprocessing fraction pools 1 and 2 on Sephadex G200 the spread of the antigenic material through the trace was much greater than expected considering that these two fraction pools had already been fractionated on Sephadex G200. This may in part have been due to molecular dissaggregation and/or complex formation during reconcentration. The volume of samples applied to the column may also have been too large.

Accordingly, simply reprocessing the fraction pools on Sephadex G200 did not result in full separation because of the tailing effect of the fractions. Thus the trace obtained on reprocessing Sephadex G200 fraction pool 1 (3.3.2.2.1.4.) still did not return to near the base line until fraction 65 (Figure 31), which was near the end of fraction pool 2 on initial fractionation (Figure 30).

There was also a further small peak around fractions 80-90 which was near the beginning of fraction pool 3 in the initial fractionation. Again the trace for reprocessing Sephadex G200 fraction pool 2 (Figure 32) continued on until fraction 85 before it returned to base line levels. This was the pattern which might have been expected from tailing.

These results indicated the presence of two main groups of antigenic molecules in the initial saline extract. The first was large molecules with HI activity and associated with gel precipitin antigen 1 and the second was a group of smaller molecules of lower HI activity associated with gel precipitin antigens 2, 3 and 4. There was more carbohydrate in relation to protein in fraction S.1.1. than in fraction S.2.2. (Figures 31 and 32). This might have indicated the composition of the molecules in these fractions but it must be remembered that these fractions were still relatively impure.

Most of the molecules which contained antigen activity in the saline extract of whole T. saginata cysticerci were excluded from Sephadex G75 (Ballad, 1973). Sephadex G75 has exclusion limits of 70,000 molecular weight for globular and 50,000 for linear molecules, therefore, the molecules in this fraction were quite large but would have included those of both size groups observed in this study. Unfortunately on reprocessing this fraction further on Sephadex G200 and DEAE Sephadex - A50, Ballad found that

most of the antigenic activity was lost. This was possibly due to dilution, as a result of the number of procedures being carried out.

The sizes of the antigenic molecules in SE were apparently of the same order as those of other taenioid cestodes. Thus the molecular weight of the two main groups of antigenic components in sheep hydatid fluid (Echinococcus) antigen were 400,000, or more, and 150,000 (Pozzuoli, Musiani, Arru, Piantelli & Mazzarella, 1972). A similar Sephadex G200 fraction of hydatid fluid from sheep infected with E. granulosus had greatest activity in the SAFA test (Gore, Sadun & Hoff, 1970). Sephadex G200 chromatography of a saline extract of T. solium cysticerci showed that the antigens concerned in haemagglutination, and six antigens with gel precipitin activity, were in a fraction with a molecular weight greater than 200,000 (Morris et al, 1968).

4.2.2.2. Ion exchange chromatography.

DE cellulose was selected as the ion exchange medium for fractionation of SE. None of the antigenically active molecules in SE were adsorbed onto CM cellulose equilibrated to 0.01M phosphate buffer pH 7.2, or if they did, they were not eluted by either acid or alkaline buffers of high molarity (3.3.2.3.1.).

The antigenically active components were adsorbed onto DE cellulose and were eluted by both acid and alkaline buffers of high molarity. Large amounts of both protein and carbohydrate were lost during the procedure, possibly during dialysis. Some of the material may have also been irreversibly adsorbed onto the cellulose, particularly in the case of carbohydrate and CM cellulose. It was noted that this might have been a useful way of removing antigenically inactive carbohydrate from SE. However, there was also a considerable drop in the antigenic activity as measured by the HI technique between untreated SE and the fractions. Possibly this drop in activity could have been accounted for in the general loss of material.

In ion exchange chromatography on DE cellulose (3.3.2.3.2.), three molarity gradients were tried before an acceptable degree of separation between the various components of SE was obtained. Gradient 1 with systems 1 and 2 (3.3.2.3.2.1. and 3.3.2.3.2.2.) illustrated that some of the material present in SE was not adsorbed onto DE cellulose equilibrated to 0.01M phosphate buffer, pH 8.0. This material was mainly carbohydrate with some protein but did not have any detectable antigenic activity. This result was also obtained in the preliminary batchwise separation (3.3.2.3.1.). DE cellulose chromatography, therefore, readily removed these molecules of little or no antigenic activity.

The presence of the level sensor with gradient 1 (3.3.2.3.2.2.) improved the separation of the fractions to some extent but it was not sufficient to separate fraction pools DE 1.2. and DE 1.3. (Figure 38) which contained the majority of the antigenic activity. Fraction pools containing detectable antigenic activity were eluted by 0.055M phosphate, 0.45M NaCl buffer, pH 8.0 and 0.1M phosphate, 0.9M NaCl buffer, pH 8.0. A great deal of material, both protein and carbohydrate, and also antigenic activity was lost. Only about 30 per cent of the protein and 20 per cent of the carbohydrate applied to the column was recovered. Some of this may have been lost by being adsorbed onto the DE cellulose or onto the walls of the tubing and collection tubes or while reconcentrating the fraction pools.

System 3 (3.3.2.3.2.3.) was a slight improvement on systems 1 and 2, but the poor separation achieved when a relatively small amount of SE was applied to the DE cellulose column, and the small amounts of material recovered suggested that 25 g of DE cellulose packed into the column may have been too great for the amount of SE applied. Therefore, after testing a third gradient (3.3.2.3.2.4.), the quantity of DE cellulose was reduced to 15g and the amount of SE applied to the column was increased. System 5 (3.3.2.3.2.5.) improved the separation of SE possibly because of this alteration in the ratio of cellulose to SE. However, the procedure did not greatly improve the percentage recovery of material (Figures 41 and 42),

suggesting perhaps that most of the material was lost during reconcentration. System 5, however, separated SE into 8 distinct fraction pools.

The main disadvantage of DE cellulose chromatography was the loss of material and hence antigenic activity. In particular gel precipitin antigens 3 and 4, which were faint in untreated SE, were often lost on fractionation. DE cellulose chromatography of SE using system 5 resulted in 8 distinct fractions (Figures 41 and 42). These results were quite reproducible and when used in conjunction with Sephadex G200 chromatography produced fraction pools of high antigenic activity from which small antigenically inactive molecules had been removed (3.3.2.3.2.6.).

Table 47 Summary of the fraction pools obtained by system 5

Fraction pool	% final buffer	Approx. molarity of elution buffer*	HI activity	Gel ppt. activity
1	8%	.08M	-	-
2	17% - 19%	.17M - .19M	-	-
3	20% - 25%	. 2M - .25M	+?	+
4	30% - 31%	. 3M - .31M	+	+
5	35% - 41%	.35M - .41M	+	+
6	50% - 90%	. 5M - . 9M	+	+
7	100%	1.0M	+	-
8	100%	1.0M	-	-

+ Antigen activity present
- Antigen activity absent

* Calculated by adding the molarity of the phosphate to that of the NaCl.

Obviously this system could be further refined but it was clearly intrinsically deficient in some ways. Molecular aggregation could easily have occurred in the relatively crude SE and this would have affected both ion exchange and gel-diffusion. In addition the original extract may simply have been too complex for ion exchange chromatography. Precipitating SE either with ammonium sulphate or TCA would have removed some of the gross contamination, but these procedures also tended to result in a loss of gel precipitin activity (3.3.1.)

Phosphate buffers are not very suitable for use with DE cellulose as phosphate is an anionic buffer and DE cellulose is an anionic exchanger. Better results might have been obtained with a cationic buffer such as Tris/HCl, but there was the problem of Tris strongly absorbing light at 220nm. However, as the first fractions of the extract with antigenic activity were not eluted until approximately 0.2M buffer was used, this problem could perhaps have been overcome. Thus 0.1M Tris/HCl buffer pH 8.0 could be used as starting buffer and 0.1M Tris/HCl pH 8.0 buffer with 1.0M NaCl as the final buffer. The problem of Tris absorbing light at 220 nm could then be overcome by adjusting the zero control on the optical unit, as the molarity of the Tris would always be constant at 0.1M. As is mentioned later other workers have used DEAE Sephadex-A50 and it might have been useful to have used this medium instead of DE cellulose so that a comparison could have been made between the results in this and other similar studies.

However, as conducted here the system did give quite a good separation of the fractions and phosphate buffers are often used with DE cellulose (e.g. Penhale & Christie, 1969). A suggested system would therefore be to use 0.1M Tris/HCl pH 8.0 as a starting buffer and 0.1M Tris/HCl 1.0M NaCl pH 8.0 as a final buffer. The gradient would remain at 0.1M Tris/HCl pH 8.0 long enough to elute any material which does not adsorb onto DE cellulose at this pH and molarity, then rise abruptly to 0.1M Tris/HCl 0.1M NaCl pH 8.0 to elute fractions 1 and 2. A slow increase in molarity would elute fractions 3, 4 and 5 at between 0.1M Tris 0.1M NaCl and 0.1M Tris 0.3M NaCl. Fraction 6 should be eluted between 0.1M Tris 0.4M NaCl and 0.1M Tris 0.8M NaCl. Fractions 7 and 8 should be eluted by the highest molarity. In view of the small fraction pool 8 it might be useful to then wash the column with 0.1M Tris/HCl, (2.0M NaCl) buffer pH 8.0 in order to elute any material still adsorbed onto the cellulose. Ultimately it may be possible to separately elute the gel precipitin antigens present in Sephadex G200 fraction pool S.2.2. by constructing a gradient using a very slow change in molarity at the point at which these antigens are eluted.

DEAE Sephadex-A50 has been used by previous authors to fractionate either extracts of T. saginata proglottids or extracts of whole T. saginata cysticerci. Stepwise elution of an extract of T. saginata proglottids using 0.1M Tris/HCl buffer, pH 8.0 and 0.1M, 0.2M, 0.3M, 0.4M and

1.0M NaCl produced 8 fractions of which 7 contained gel precipitin antigens (Grossklaus & Walther, 1970). The fourth fraction contained 3 gel precipitin antigens and may therefore, have corresponded to fraction DE5.4a. (Figure 41) and DE5.4b. (Figure 42) eluted from the DE cellulose in this study.

Unfortunately most of the antigenic activity was lost when the first fraction from Sephadex G75 chromatography of an extract of whole T. saginata cysticerci was fractionated on DEAE Sephadex-A50 (Ballad, 1973). A potassium/sodium buffer (molarity not stipulated) pH 6.5 was used with an NaCl gradient increasing linearly from 0.001M to 1.5M NaCl. The loss of activity may have been due to the fractions becoming too diluted.

4.2.2.3. Immunoadsorption.

The procedure was introduced late in the study and it was not possible to carry out a great deal of work with it. However the results obtained suggest that this technique offers a valuable method for purifying T. saginata antigens.

The loss of activity in the fractions obtained in experiment 1a (3.3.3.1.1.) indicated that great care has to be taken when handling these purified fractions, which contain such small amounts of material. The addition of 5 per cent albumin to these fractions (3.3.3.1.2.) although perhaps effective in preventing denaturation,

was not very practicable as the albumin may have interfered with the assay technique. The results from experiment 2 (3.3.3.2.) indicated that immunoadsorbents used in series can at least partially remove some of the cross reacting antigens and that these further purified antigens are effective when used in the ELISA technique.

Packing the immunoadsorbents into small chromatographic columns (3.3.3.3.) proved more convenient than batchwise methods. The addition of cellulose fibres to the insolubilised serum was an effective means of preventing the insolubilised serum compacting in the columns which was a recurring problem in this study. The glycine/HCl buffer pH 2.8 containing 2.5M NaCl was a satisfactory elution buffer for chromatographic columns. The 0.05M Tris/HCl buffer pH 9.0 containing 5M NaI, which was the best elution buffer used with the batchwise procedure, was not satisfactory when used in column immunoadsorption because it appeared to affect the immunoadsorbent in such a way as to make it compact even more readily than usual. It was interesting to note that once purified, the T. saginata antigen (F4) was mainly protein with less than 20 per cent carbohydrate.

Purification of SE by immunoadsorption reduced the 'non-specific' ELISA readings obtained with serum from slaughter house cattle, however, there was no net increase in the sensitivity of the technique. In fact the ELISA readings with untreated SE were higher than those obtained with the purified antigen extracts. These results are similar to those reported on the purification of E. granulosus antigens (Pozzuoli et al, 1974) who also reported no net sensitivity

gain but improved specificity when antigens purified by immunoadsorption techniques were used in serology.

Further aspects of the potential use of immunoadsorption techniques are discussed later (4.9.)

4.3. Serological techniques.

4.3.1. The MGP technique.

The MGP technique (2.7.5.) allowed an estimate to be made of the number of antigen/antibody reactions occurring in a sample. However, the technique lacked sensitivity and it was often difficult to see the gel precipitin lines in the agar. This difficulty was overcome to some extent by 'developing' the lines either with cadmium acetate or by overlaying the agar with rabbit anti-bovine serum.

4.3.2. The IDH technique.

The IDH technique (2.7.6.), conducted on a micro scale proved to be very efficient. The main advantages of the micro technique were the amount of time saved carrying out the technique over the macro form and also the saving in the amounts of reagents used.

4.3.3. The HI technique.

The HI technique as conducted here (2.7.7.) proved to be a useful and sensitive procedure for monitoring the antigenic activity in fractions eluted from either ion exchange or gel filtration columns.

4.3.4. The ELISA technique.

From a short comparison of micro and macro-ELISA using both AP and HRPO as indicator enzymes it was concluded that there is not a great deal of difference in the performance of the various forms of this technique (3.2.13.). A more important consideration was the time involved. If a large test with many samples is being performed it is difficult to carry out all the necessary steps in time if the incubation times in serum and conjugate are short. The longer incubation times are, therefore, preferable and in this work incubation was standardised at 4 hours in serum and 18 hours in conjugate. Some other workers favoured shorter incubation periods of 30 minutes in serum and 30 minutes in conjugate at 37°C, (Ruitenberg, Steerenberg & Brozi, 1975 and Voller Bartlett & Bidwell 1976), whereas others also adhered to the longer incubation times at room temperature (Bout, Dugimont, Farag & Capron, 1975) as used in this study.

The obvious advantage of micro-ELISA over the macro form of the techniques is that the former saves on reagents and the time taken for the washing procedure. Micro-ELISA is possibly better suited to automation and the equipment for this should shortly be available commercially (Dinatek Micro-ELISA systems, Dynatak Ltd., England).

The enzyme indicator used in the ELISA technique is worth consideration. Of the two enzymes commonly used, AP and HRPO, AP has the advantage that the colour produced

by the reaction with the substrate is stable once the reaction has been stopped with NaOH. The yellow colour produced is, however, not very dense. The brown colour produced by HRPO and its substrate is much more suitable for visual assessment. Unfortunately this colour is not always stable and the tubes visibly darken if left for some time e.g. overnight at $4^{o}C$. This colour change may be due to oxidation of the 5AS. As a financial consideration, AP and its substrate are considerably more expensive than HRPO and its substrates. Conjugates labelled with HRPO are available commercially (Nordic Immunochemical Laboratories Ltd.). In this work, unless otherwise stated, macro-ELISA with alkaline phosphatase as the indicator enzyme was used.

The results (3.2.11.) showed that possibly some variation existed in the ELISA readings obtained with different batches of conjugate. This illustrated an aspect of the use of enzyme conjugates which is in need of standardisation. Unfortunately, the necessary techniques were not developed for this study. Possibly one of the best ways to standardise and characterise the enzyme conjugates is as suggested by Engvall Perlmann (1971). The amount of globulin in a conjugate was estimated by a radioimmunosorbent technique (RIST) (Wide & Porath, 1966), but any of the currently available radioimmunosorbent techniques (Hunter, 1974) could be used. The enzyme activity (in this case AP) was determined by following the hydrolysis at room temperature ($22^{o}C$ - $24^{o}C$) of 2.5 mM p - nitrophenyl phosphate (NPP) in

0.05M carbonate buffer pH 9.8, containing 1 mM $MgCl_2$. The specific activity of the conjugate can then be expressed as the increase in the absorbance at 400 nm per minute per µg of globulin (A_{400}/min/µg globulin). A similar procedure could equally well be developed for HRPO.

At present even commercially produced conjugates are not characterised in this manner, but it would facilitate the standardisation of the ELISA technique if all conjugate preparations were described in these terms.

4.3.5. The SAFA technique.

It was unfortunate that the SAFA technique was so insensitive. This insensitivity may in part have been due to the fact that only a conversion of a spectrofluorimeter was available. The use of a purpose built fluorimeter might have given somewhat better results.

There are perhaps other ways in which the technique could be improved. It was found that black cellulose acetate discs gave no readings on the spectrofluorimeter (3.1.1.), at least as the apparatus was set up for this study.

One possibility is that the fluorescein was present within the fabric of the discs rather than on the surface and the black discs were absorbing all the emitted light so that none could register on the spectrofluorimeter scale. However, as pointed out by Daniellson (1965) white

cellulose acetate filter paper exhibits autofluorescence. The spectrofluorimeter was set up and adjusted using the filter combination selected for use with white discs, and this could have made it insensitive to the relatively low light emission by fluorescein coated black discs. The autofluorescence of these white discs may have been the cause of the "extraneous light" originally assumed to have come from the monochromator (2.7.12.1.). This "stray light" was the main reason why so many filters had to be placed in the fluorimeter and this resulted in sub-optimal conditions for measuring fluorescence. The use of black discs with the correct filter combination is probably worth investigating. However, the apparatus did appear to be capable of measuring fluorescence on the white filter paper discs and these white discs were used in SAFA.

4.3.6. A comparison of the SAFA, ELISA and IDH techniques.

SAFA lacked in sensitivity as compared to IDH (3.1.7.), but this could be overcome to some extent by the four layer technique (3.1.8.). ELISA compared favourably in sensitivity with IDH (3.2.6.) and was consequently much more sensitive than SAFA. ELISA had other advantages over SAFA. In ELISA the colour reaction of the indicator enzyme with its substrate could either be assessed visually or by a spectrophotometer or colorimeter. The fluorescence of the SAFA discs could not be assessed visually, and a fluorimeter was required to read the results. This is an

expensive and specialised piece of apparatus. Handling the ELISA tubes or micro-ELISA plates and small volumes of liquid was easier and less time consuming than handling the SAFA discs.

The relatively poor results obtained with SAFA, as compared with ELISA, are not restricted to the T. saginata system in cattle. Direct comparisons of the two techniques have not been found in the literature, but ELISA and SAFA have been applied separately to the sero-diagnosis of some other parasitic infections. However it was often not possible to compare the tests directly because of the different methods used to express the results. In experimental infections of T. spiralis mice, rats and rabbits (Gore & Sadun, 1968b) the SAFA test first gave a positive reaction 2 weeks pi whereas an antibody response could be detected with ELISA in pigs experimentally infected with T. spiralis only 3 days pi (Ruitenberg, Steerenberg, Brozi & Buys, 1974). In the SAFA test for human echinococcosis (Gore, et al, 1970) most of the positive sera had titres of around 1:160, whereas in the ELISA test for this infection (Bout et al, 1975) the optimum dilution of serum was 1:500.

This is not to say that ELISA used in the T. saginata system in cattle compared favourably in sensitivity with the same assay used with other helminths or parasitic infections. The optimum serum dilution for macro-ELISA in the T. saginata system was only 1:50. This compares with 1:500 for human echinococcosis (Bout et al, 1975) and

1:1,600 for human T. cruzi infections (Voller et al 1975). However the optimum dilution for serum from Schistosoma mansoni infected patients was 1:100 (Deelder, Ruitenberg, Kornelis & Steerenberg, 1977). The ELISA technique was more convenient than SAFA and had more potential for field use so this was selected for further development.

4.4. The relative activities of Sephadex G200 fractions of SE in various serological techniques.

The Sephadex G200 fractions of SE did not all have the same activities in the various serological techniques (Table 48). In the HI technique all the Sephadex G200 fraction pools were able to inhibit haemagglutination (3.3.2.2.1.3.) to some extent but in the IDH technique (3.4.1.2.) only Sephadex G200 fraction pool 1 coated tanned red blood cells would haemagglutinate in the presence of positive antiserum. This may suggest that some, or even most, of the tailing in haemagglutinin activity is caused by the presence of antigen fragments. The first Sephadex G200 fraction of an extract of whole T. saginata cysticerci also had activity in the IDH test (Bol'shakova et al, 1975).

Purifying SE by gel filtration removes many non-specific molecules which may compete for attachment sites on the tanned red blood cells. This may account for the slightly improved IDH titres using Sephadex G200 fraction pool 1 as compared to SE (3.4.1.2.). It is surprising that there was no IDH activity in fraction pool 2 at all,

because even if the molecules in this fraction pool had no haemagglutinating activity themselves, there would probably have been a "tail" of active molecules from fraction pool 1. Presumably, if they were present, there was not a sufficient concentration to allow haemagglutination. Again, the presence of fragments of the large haemagglutinin molecules active in the HI technique but not the IDH technique, in the tail from Sephadex G200 chromatography may account for this lack of activity. Gel precipitin activity was concentrated in Sephadex G200 fraction pool 2 (3.3.2.2.1.) but there was at least one gel precipitin antigen in fraction pool 1. Both the ELISA and SAFA techniques appear capable of detecting antibodies in sera to the antigens in both Sephadex G200 fraction pools 1 and 2 (3.4.1.1.2.1. and 3.4.1.2.). There was also a little antigenic activity in fraction pool 3 but this was probably a tail from fraction pool 2.

Table 48 The relative activities, expressed as a percentage of the activity of SE, of Sephadex G200 fractionated extracts of SE used in various serological techniques.

Serological technique	SE	2.S.1.	2.S.2.	2.S.3.
HI	100	95	56	12
IDH	100	142	0	0
MGP	1,2,3,4	1,2	1,2,3,4	2
ELISA	100	81	92	33
SAFA	100	140	60	40

Purifying SE by Sephadex G200 chromatography did not increase the sensitivity of the ELISA and SAFA techniques. Sephadex G200 fraction pool 1 contained mainly haemagglutinins whereas fraction pool 2 contained mainly gel precipitins. Techniques such as SAFA and ELISA should be able to measure quantitatively the amount of antibodies (to both groups of antigens) in the sera of T. saginata infected cattle. However ELISA was used in preference to SAFA because it was the more sensitive of the two techniques.

The ELISA technique works by fixing the antigens under study onto the walls of plastic tubes and according to Deelder et al, (1977) both proteins and carbohydrates adsorb onto the plastic. Presumably something similar happens in SAFA when the antigens are adsorbed onto the cellulose acetate discs. Therefore, with these techniques, so long as the antigen under study is adsorbed onto the matrix, the antigen/antibody reaction can carry on unhindered. Factors such as the size of the antigen molecules (apparently important in the IDH test) should not interfere with the reaction.

It should be possible to absorb all the HI activity from a sample of positive serum using Sephadex G200 fraction S.1.1. which is rich in haemagglutinins but leave most of the gel precipitin activity in the serum. The results (3.4.1.1.2.2.) partially agree with this hypothesis in that S.1.1. fraction was more inhibited than either crude antigen or fraction S.2.2. However, the results are not very conclusive and emphasise the difficulty of absorbing sera with

relatively uncharacterised soluble antigen samples such as fraction S.1.1., when using sensitive techniques such as ELISA to monitor the results. As was outlined previously (4.2.2.3.) alternative methods of immunoadsorption exist which do not result in such complex and ill defined mixtures of serum and antigen.

4.5. Larval and somatic antigens used in ELISA.

Calves orally infected with T. saginata eggs gave a serological response of the same magnitude when either SE, a saline extract of T. saginata cysticeral scolices and membranes, or T. saginata cyst fluid were used as antigen in the ELISA technique (3.4.1.1.1.). However, calves given a series of intra-muscular injections of an homogenate of T. saginata proglottids did not give a good serological response when T. saginata cyst fluid was used as antigen. Similarly, with orally infected calves given an intramuscular injection of an homogenate of T. saginata proglottids, an increased serological response was detected when an homogenate of T. saginata proglottids was used as antigen in the ELISA technique. A response was not detected when T. saginata cyst fluid was used as antigen (3.4.2.1.4.). These results suggest both that the serological response to infection differs quantitatively to that following immunisation and that there are differences in the immunogenic and antigenic components of the somatic and cyst fluid extracts.

However, Machnicka (1974) found cross reacting antigens present in T. saginata cyst fluid, an extract of T. saginata cysticercal scolices and membranes and an extract of T. saginata proglottids. This author used antisera from rabbits immunised by intramuscular injections of the various antigens so it may be that, although there are common immunogenic components in these different extracts, orally infected cattle do not respond well to them, perhaps because when orally infected they are not normally exposed to these antigens. On the other hand the same calves given intramuscular injections of a homogenate of T. saginata proglottids (3.4.1.1.1.) had previously given a gel precipitin response to cyst fluid (Gallie & Sewell, 1976). It is therefore possible that the antibody response to the cyst fluid antigens had tailed off in these cattle before these particular serum samples were taken. Although the gel precipitin test gives a reasonable indication of the number of antigen/antibody reactions occurring in a system, it is not a very good indicator of the level of the antibody response.

The two calves given intramuscular injections of hatched and activated T. saginata onchospheres (3.4.2.3.) gave different serological responses, as measured by the ELISA technique. Calf E4 only showed a serological response to infection when SE was used as antigen in the ELISA technique. Calf E5 gave a serological response with both SE and T. saginata cyst fluid antigens. Most

of the onchospheres must have died in calf E4 before developing into cysticerci. Only 9 cysts were found in the carcase, therefore, the calf will mainly have been exposed to "onchospheral" antigens. One hundred and seventy one cysts developed in calf E5, and this calf had therefore experienced both onchospheral antigens and more fully developed cysticerci in its tissues. This may explain the different results.

Some cyst fluid or metabolic by-products must escape from the cysts and stimulate the serological response of the calves as is the case with orally infected calves showing a serological response to cyst fluid antigens. However neither of these calves given intramuscular injections with hatched and activated T. saginata onchospheres gave a very strong serological response to infection. This was also found by Gallie & Sewell (1976) who used the IDH and gel precipitin techniques to monitor the serological response. ELISA, however, first gave a positive reaction by 5 - 6 weeks pi, fractionally earlier than the IDH technique which took 8 weeks (Gallie & Sewell, 1976).

It is interesting that these calves given intramuscular injections with hatched and activated onchospheres should show such a poor serological response to infection. An antigenic stimulus must be present since calves treated in this way develop an immunity to oral infection with T. saginata eggs (Gallie & Sewell, 1976). These authors suggested that serological tests do not demonstrate the presence of

protective antibodies against the tissue phase of this parasite. This may suggest the presence of a cell mediated response against the invasive onchospheral stage although there is also evidence of passive transfer of resistance to infection with T. saginata in neonate calves using antiserum from experimentally immunised cattle (Lloyd & Soulsby, 1976).

4.6. Experimental oral infection of older calves with T. saginata eggs.

ELISA compares favourably with IDH for monitoring the serological response of calves (3 month - 1 year old) to experimental oral infection with T. saginata eggs (3.4.2.1.1.). Ruitenberg et al (1974) reported that in pigs experimentally infected with T. spiralis a positive serological response could be detected with ELISA by 3 days pi. However, T. spiralis larvae are infective by day 17 pi, whereas T. saginata cysticerci are not infective until 10 - 11 weeks pi (McIntosh & Miller, 1960). The ELISA technique registers a positive serological response within 2 - 4 weeks pi, well before the larvae are at the infective stage.

In calves orally infected with T. saginata eggs, the gel precipitating antibodies could be detected by 2 weeks pi, the response remaining strong for up to $6\frac{1}{2}$ to $7\frac{1}{2}$ months pi. In contrast, haemagglutinating antibodies were not detected until 4 - 5 weeks pi and took 6 months to reach peak titres (Gallie & Sewell, 1974b). Sephadex G200 fraction pool 1

is rich in haemagglutinins whereas Sephadex G200 fraction pool 2 contains mainly gel precipitins (3.3.2.2.1.3.). The ELISA results obtained with these two fractions and untreated SE (3.4.2.1.3.) indicate that ELISA can be used as a quantitative technique to detect both these types of antigen molecules. Indeed the ELISA technique could probably be used to detect antibodies against any specific antigens if these could be produced by further purification techniques.

4.7. Calves neonatally infected with T. saginata eggs.

The preliminary work with neonate calves (3.4.2.2.1.) indicated that the serological response to infection was very weak and consequently lower dilutions of sera were used when testing the sera more fully. During the first 2 - 3 months the control calves had higher ELISA values than in latter months (3.4.2.2.2.). This may have been due to maternally transmitted colostral globulins cross reacting with the antigen in the ELISA technique (SE). All these calves were given colostrum before leaving their dams and colostral globulin can persist in the sera of calves for several weeks (Herbert, 1970).

Not all the calves in the experimental groups gave a good serological response to infection and the extent of the response was not related to the presence or absence of T. saginata cysticerci from the original oral infection (Gallie & Sewell, 1974a). This experiment thus

replicates the situation that occurs in East Africa, where calves with a poor serological response to infection with T. saginata may be resistant to reinfection with T. saginata eggs but still harbour viable cysts in the musculature (Soulsby, 1963).

It is difficult to see how any immunological techniques which simply measure serum antibody levels would be of diagnostic value in these circumstances since some of the calves hardly responded at all. Even if highly purified antigens were developed for ELISA, the results from partial purification of the extract of T. saginata proglottids (3.4.1.) suggest that no net increase in sensitivity can be expected by the use of purified extracts and that only the specificity of the tests is improved. ELISA, as has been pointed out by many authors, is a very sensitive test consequently these young cattle cannot be giving an antibody response to infection with T. saginata. There is the possibility that ELISA could be used to detect a difference in the serological response of tolerant and uninfected calves to immunisation of T. saginata antigens - i.e. an indirect test.

In contrast, older cattle do respond to infection with T. saginata with an antibody response which can be monitored by ELISA. Perhaps ELISA used to measure serum antibodies would mainly be of diagnostic use in testing for older cattle infected with T. saginata eggs. Such a situation occurs in the U.S.A. (Dewhirst et al, 1976). However, the problems encountered with cross reacting antigens would have to be overcome first.

4.8. Farm cattle.

The serum protein levels of the cattle from the three Fife farms (3.4.3.1.) probably more closely reflects the general status of the herds rather than the probability of the calves being infected with T. saginata. The two farms in Fife gave similar results whereas the farm in Lothian gave different values for both total protein and globulin concentration.

Calves experimentally infected with T. saginata eggs have increased protein levels in their serum in the period from 1 - 11 weeks pi (Gallie & Sewell, 1974b). However, the cattle on farm A, on which infection occurred, actually had less protein in their serum than the cattle on the control farms. The cattle on farm A were serum sampled about 7 months pi so the difference in serum protein levels is unlikely to have been caused by T. saginata infection. The sera from cattle on farm A showed hypo-albinunaemia as compared to the two control farms, a pattern similar to that reported in experimental cattle (Gallie & Sewell, 1974b) but it is unlikely that this is related to T. saginata infection alone. Hyper-globulinaemia has been reported in calves experimentally infected with T. saginata (Evranova & Mosina, 1965, Alferova, 1968 and Gallie & Sewell, 1974b). However, the globulin concentration in the sera of cattle on farm A was little more than that of the sera from control farm B and indeed much less than that of the sera from control farm C.

With the IDH and SAFA techniques farm A and B (Fife) gave similar results whereas farm C (Lothian) gave lower readings or titres, (3.4.3.1.4. and 3.4.3.1.6.). The micro-gel precipitation technique (3.4.3.1.5.) although effective for monitoring the response of cattle to experimental infection with T. saginata, is probably too insensitive for field studies involving light infection. Similarly poor results were obtained with the gel precipitation reaction in cattle in Chad (Martin, 1972).

With the ELISA technique and for unknown reasons two of the cattle on control farm B gave very high readings. For the purpose of analysis these two outliers were either omitted or included in the calculations. When they were omitted both the control farms B and C gave a similar pattern for the different antigen samples (3.4.3.1.7.). The untreated SE and fraction S.1.1. gave similar readings while fraction S.2.2. gave significantly higher readings. This pattern was not repeated on the infected farm A. The untreated SE gave significantly lower readings than either antigen S.1.1. or S.2.2. and there was no significant difference between the results for fractions S.1.1. and S.2.2.

Fraction S.2.2. may have given increased readings partly due to the presence of non-specific antigens whereas fraction S.1.1. gave increased readings with the T. saginata infected cattle. However, the two high titre control sera on farm B indicated that fraction S.1.1. can also cross

react with sera from cattle with no history of T. saginata infection and therefore, the test is not reliable. Fraction S.1.1. may, be the more suitable antigen preparation for further purification, as fraction S.2.2. apparently contained more non-specific components reacting with control sera as well as sera from infected calves. However, even here further purification may still remove the non-specific components.

From the results with the Dutch sera, it was not possible to say that the cattle had serum antibodies to T. saginata present at the time the samples were taken, which was late on in the infection. ELISA can not therefore, as yet be regarded as a reliable serological test in field studies for T. saginata infection. In a field trial on 1022 slaughter pigs (Ruitenberg, Steerenberg Brozi & Buys, 1975), ELISA failed to detect serum antibodies attributable to T. spiralis infection but a digestion method for the detection of T. spiralis larvae was also negative. However, despite these poor field results ELISA remains a sensitive and useful tool for monitoring the antibody response of cattle to experimental infection with T. saginata.

4.9. General Discussion.

The results from the 3 Scottish farms and the Dutch farm suggest that the antigens prepared by Sephadex G200 gel filtration were not pure enough for field work, because of cross reactions particularly on farm B. Unfortunately due to a lack of material, it was not possible to test the

fraction of SE purified by ion exchange chromatography with this sera nor were fractions of SE purified by immunoadsorption tested with these sera.

If antigens are purified by gel filtration or ion exchange chromatography, although the extract is separated out into its component parts, it is really just a matter of chance whether the immunologically active molecules are separated from non-specific and/or cross reacting components. In addition the presence of these non-specific components acts to confound the efficiency and reproducibility of both these fractionation procedures. Furthermore after fractionation of the extract, there is the time consuming problem of assessing all the different fractions.

Immunoadsorption techniques are beginning to be used in the purification of helminth antigens and these techniques should provide a way of preparing very specific antigens, since these methods rely on the specificity of the antigen/antibody reaction. The immunoadsorption work carried out in this thesis suggests that cross reacting and other non specific components could indeed be removed from SE or indeed any extract of T. saginata material by using a combination of the appropriate immunoadsorbents.

Extracts produced by immunoadsorption could then be further purified by gel filtration and ion exchange chromatography using the information already gained, to isolate the two types of antigens present in SE i.e.

the large molecular weight haemagglutinins and the smaller gel precipitins. One difficulty with this work would be preventing the purified or semi-purified antigens from polyimerising or denaturing, which they may readily do, thus complicating the purification procedure (Pozzuoli et al, 1975).

The main disadvantage of these purification procedures is that they are costly in terms of time and loss of material. The purification of a large quantity of crude extract would result in only tiny quantities of the specific antigen. This is where the recently developed, sensitive techniques such as ELISA and DASS, which require miniscule quantities of antigen present a clear advantage. Although, provided the non-specific absorption problem can be overcome in theory there would be as much specific antigen in the purified product as in the original crude product. Once purified it could well be protected by an inactive carrier such as albumin.

There are a number of ways in which immunoadsorption techniques could be used in the purification of helminth antigens. For example, these could be used as a precursor to conventional chromatography, which might reduce the complexity of the sample to be fractionated. This may lead to a better chromatographic separation of the antigenically active components of the helminth extract.

Immunoadsorption techniques also have some potential for use in the large scale purification of helminth antigens. Once the antigens have been isolated, hyper-immune antisera could be produced against them either using rabbits or other hosts. Immunoadsorbents could then be prepared from this antisera for use in the purification of saline extracts of the helminth.

The epidemiology of T. saginata has recently been reviewed (Gemmel & Johnstone, 1977). It is a very complex issue and its clarification is complicated by the costs involved when experimenting on cattle. However, the situation is gradually becoming clearer.

Gallie & Sewell (1974a) found that the resistence of calves neonatally infected with T. saginata is not necessarily associated with a strong serum antibody response. Although a group of serially infected calves were resistant to reinfection the mean haemagglutination titre of that group was no higher than that of a singly dosed group. These results were also found when the sera from these calves were tested by ELISA (3.4.2.2.2.). More importantly when looked at individually one of the calves in the serially dosed group hardly responded at all, despite the fact that it was resistant to challenge infection. Gallie & Sewell (1974b) therefore concluded that the serological response to infection shown by 3 month old cattle, as detected with the presently available antigens, is not necessarily protective.

Older cattle infected i/m with hatched and activated T. saginata ova gave only a very slight serological response (Gallie & Sewell 1976 and 3.4.2.3.). However the presence of only 9 T. saginata cysticerci in one calf could be detected by ELISA. Therefore, there must be some, though slight, antigenic stimulation caused by the presence of T. saginata ova or cysticerci in the musculature and indeed their presence is sufficient to produce immunity to oral challenge with T. saginata eggs (Wickerhauser et al, 1971, Wickerhauser Žukovic, Džakula, & Maran, 1974 and Gallie & Sewell, 1976), even though the serological response is low. The factors involved in this immunity are probably in some way related to the excretory/secretary (ES) antigens, similar to those used by Rickard & Bell (1971 a, b & c) to induce immunity in lambs to T. ovis infection.

This evidence of resistance to oral infection in the presence of little or no antibody response to infection may suggest that protective immunity in these calves is due to a cell mediated response to oral challenge. This is one feature of the immunology of T. saginata infection in cattle which has still to be fully investigated, although the application of cell mediated techniques to field work would certainly cause problems.

Kagan (1974) pointed out that until recently most immunological tests in helminth infections have been used to look for a serum antibody response to infection. There are few reports in the literature of immunological techniques

being used to detect circulating antigens in helminth infections. The presence of circulating antigens in the sera of mice infected with S. mansoni has been known for some time (Berggren & Weller, 1967) and Nash, Prescott & Neva (1974) characterised the antigen as a polysaccaride. Voller et al (1976) described a double antibody sandwich method of ELISA and used it to detect this antigen in the sera of infected patients. However they indicated that the results should be interpreted with caution.

If a similar ELISA double antibody sandwich technique could be developed for use in bovine cysticercosis, then the problem associated with neonatally infected calves may be overcome. Even if calves did not give a strong humoral response to infection, circulating antigens might still be detectable if live T. saginata cysticerci were present in the musculature secreting ES antigens. Indeed if T. saginata antigens can be produced in sufficient quantity and of an acceptable purity then it would be possible to use an ELISA competitive immunoassay as described by Engvall Johsson & Perlmann (1971) to detect circulating antigen. This technique was reported to be very sensitive and to detect 1 - 100ng/ml quantities of antigen. A radioimmunosorbent assay such as described by Catt & Tregear (1967) could also be used but according to Engvall, Johsson & Perlmann (1971) this technique is no more sensitive than the ELISA competitive immunoassay.

In tolerant animals circulating antigens are removed from the system slowly by normal catabolic mechanisms, polysaccaride antigens in particular being removed very slowly (Herbert 1970). There is some reason to believe that at least two of the antigens which elicit an antibody response to T. saginata in older cattle are carbohydrate in nature (3.3.1.1.). Therefore, if these are present as circulating antigens, they would be detectable for some time in tolerant calves. This of course is only speculation and there may be many pitfalls. For example, the circulating antigens (if present) may only be there for a limited time. However, in view of the poor antibody response in these neonataly infected calves, a search for the existence of circulating T. saginata antigens may be worthwhile.

The potential advantages of the ELISA technique both for experimental and field work have already been outlined (Ruitenberg, Steerenberg, Brozi & Buys, 1975 and Voller et al, 1976). The main advantages of the technique are its sensitivity and the ease with which the results can be assessed, either by visual inspection or by reading the results on a spectrophotometer or colorimeter, which gives objective readings. The technique itself is simple to perform and if conducted on a microscale using 30 minute incubation periods it can be very rapid. It is not very expensive, can be conducted very simply as a manual technique but has great potential for automation. Ruitenberg et al (1974) reported that 4,000 samples/day could be

processed with macro-ELISA even using semi automatic equipment. The reactants used are relatively stable but they can be stored lyophilised if this is necessary. The technique also employs soluble antigens which allows them to be purified by physico-chemical or other purification techniques. These potential advantages apply equally well if ELISA is conducted as a double antibody sandwich technique or to detect serum antibodies. The main disadvantage of ELISA is that if conjugate is not readily available commercially, its preparation requires some considerable time and effort.

Deelder et al (1977) concluded that DASS was not as convenient as ELISA for use in the field. They also considered that polysaccaride antigens were unlikely to be adsorbed onto the CNBr activated Sepharose 4B beads, but this difficulty could perhaps be overcome by using CNBr activated Sepharose 4B - Con A. The main advantages of DASS is that the reaction product is a stained precipitate and the coloured beads are easily seen. As suggested by Deelder et al (1977), by using different sized beads coated with different antigens, several tests for different antibodies could be carried out at once on a small volume of serum. Indeed the possibility arises by using DASS or an adaptation of it to test for both circulating antigen and antibody in the same serum sample. Different sized Sepharose beads could be coated either with antigen or a specific antibody for a double sandwich ELISA test. After incubation in the serum sample, a mixture of the appropriate

conjugates could be added and the results assessed by visual examination with the aid of a microscope.

These recent advances have thus resulted in several more techniques to be investigated in the search for a reliable means of detecting T. saginata infection in naturally infected cattle, especially in the complicated epidemiological situation occurring in areas such as East Africa.

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APPENDICES

Appendix 1a. Table 7 – Comparison of method of drying SAFA discs

Method of drying SAFA discs	Spectrofluorimeter readings at an excitation wavelength of 465 nm	
	Positive serum (E24)	Normal serum (N10)
30 minutes at 37°C	0.10	0.02
	0.08	0.02
	0.10	0.02
	0.08	–
Overnight at room temperature	0.09	0.01
	0.11	0.04
	0.09	0.02
	0.10	–

Readings are adjusted for the saline control reading.
Results in Table 7 are expressed as the mean and standard deviation of these readings.

Appendix 1b. Table 8 – Effect of various fixatives on the sensitivity of SAFA

Fixative	Spectrofluorimeter readings at an excitation wavelength of 465 nm						
	Positive serum (E24)				Normal serum (N10)		
Air dried	0.15	0.14	0.17	0.13	0.03	0.02	0.03
Formalin	0.13	0.15	0.14	0.09	0.02	0.03	0.01
95 *per cent* ethyl alcohol	0.09	0.04	0.11	0.09	0.01	0.00	0.00
1 *per cent* acetic acid	0.09	0.12	0.09	0.1	0.01	0.01	0.01

Readings are adjusted for the saline control reading.
Results in Table 8 are expressed as the mean and standard deviation of these readings.

Appendix 1c. Table 9 – Time of incubation of antigen/antibody complex

Time of incubation of Ag/Ab complex	Spectrofluorimeter readings at an excitation wavelength of 465 nm							
	Positive serum (E24)				Normal serum (N10)			
30 minutes	0.09	0.06	0.10	0.08	0.02	0.03	0.01	0.00
45 minutes	0.08	0.07	0.09	0.08	0.02	0.01	0.00	0.00
60 minutes	0.09	0.09	0.10	0.08	0.01	0.00	0.02	0.03

Readings are adjusted for the saline control reading.
Results in Table 9 are expressed as the mean and standard deviation of these readings.

Appendix 1d. Figure 17 – Antigen titration for the SAFA technique

Antigen concentration μg/ml protein	Spectrofluorimeter readings at an excitation wavelength of 465 nm															
	Positive serum (E24)								Normal serum (N10)							
	1:2.5				1:5				1:2:5				1:5			
100	0.18	0.13	0.19	0.13	0.10	0.06	0.15	0.13	0.02	0.00	0.02	0.07	0.00	0.00	0.01	0.02
50	0.10	0.07	0.19	0.11	0.12	0.08	0.15	0.11	0.00	0.00	0.02	0.02	0.00	0.00	0.01	0.00
25	0.00	0.08	0.13	0.10	0.06	0.00	0.11	0.08	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00
12.5	0.01	0.03	0.11	0.08	0.04	0.00	0.06	0.04	0.00	0.00	0.03	0.02	0.00	0.00	0.00	0.00
6.25	0.00	0.01	–	0.06	0.01	0.04	–	0.03	0.00	0.00	–	0.01	0.00	0.00	–	0.00

Readings are adjusted for the saline control reading.
Results in Figure 17 are expressed as the mean of these readings.

Appendix 1e. Figure 18 – Conjugate titration for the SAFA technique

Dilution of FITC conjugate	Spectrofluorimeter readings at an excitation wavelength of 465 nm							
	Positive serum (E24)				Normal serum (N10)			
1:12.5	0.17	0.19	0.21	0.2	0.04	0.04	0.05	0.03
1:25	0.14	0.15	0.15	0.14	0.03	0.04	0.04	0.01
1:50	0.12	0.12	0.12	0.12	0.01	0.00	0.02	0.02
1:100	0.09	0.08	0.06	0.07	0.01	0.00	0.02	0.02

Readings are corrected for the saline control reading
Results in Table 18 are expressed as the mean reading.

Appendix 1f. Figure 19 – Serum titration for the SAFA technique

Dilution of serum	Spectrofluorimeter reading at an excitation wavelength of 465 nm			
	Positive serum (E21)		Normal serum (N10)	
1:5	0.10	0.11	0.00	0.00
1:10	0.08	0.13	0.01	0.00
1:20	0.05	0.06	0.02	0.00
1:40	0.00	0.04	0.00	0.02
1:80	0.01	0.02	0.02	0.00
1:160	0.00	0.01	0.00	0.00

Readings are corrected for the saline control reading
Results in Figure 19 are expressed as the mean of these readings.

Appendix 1g. Table 11 – Comparison of four layer technique with the indirect SAFA technique

Test employed	Spectrofluorimeter readings at an excitation wavelength of 465 nm						
	Positive serum (E24)				Normal serum (N10)		
Four layer	0.25	0.23	0.25	0.24	0.04	0.03	0.06
Indirect	0.15	0.13	0.12	0.13	0.01	0.02	0.02

Readings are corrected for the saline control reading
Results in Table 11 are expressed as the mean and standard deviation of these readings.

Appendix 1h. Table 12 – SAFA controls

Control name	Spectrofluorimeter readings at an excitation wavelength of 465 nm			
Positive serum (E24) control	0.12	0.10	0.12	0.10
Antigen control	0.03	0.03	0.025	–
Conjugate control	0.01	0.01	0.02	–
Normal serum (N10) control	0.04	0.04	0.05	–
Antigen control	0.00	0.01	- 0.02	–
Conjugate control	0.00	0.00	0.00	–
Saline control	0.03	0.025	0.01	–
Antigen saline control	0.00	0.00	- 0.01	–
Saline conjugate control	0.01	0.00	0.00	–

Results are expressed in Table 12 as the mean and standard deviation of these readings.

Appendix 2a. Figure 20 – Stability of antigen coated ELISA tubes

Weeks ELISA tubes were stored	ELISA readings at 400 nm Serum E24 1:50			E24 1:500			N10 1:50			N10 1:500		
1	0.292 0.277	0.347 0.282	0.307	0.142 0.217	0.217 0.222	0.197	0.047 0.032	0.022 0.012	0.032	0.052 0.022	0.027 0.017	0.022
2	0.286 0.341	0.381 0.381	0.371	0.211 0.236	0.251 0.286	0.286	0.036 0.036	0.046 0.056	0.051	0.041 0.031	0.016 0.031	0.036
3	0.240 0.265	0.275 0.285	0.255	0.080 0.170	0.175 0.08	0.165	0.040 0.050	0.035 0.030	0.025	0.020 0.015	0.015 0.025	0.025
4	0.195 0.185	0.185 0.175	0.215	0.160 0.165	0.150 0.170	0.150	0.050 0.040	0.025 0.035	0.030	0.040 0.040	0.025 0.030	0.040

Readings are corrected for the saline control reading.
Results in Figure 20 are expressed as the mean of these readings.

Appendix 2b. Figure 21 – Antigen titration for the ELISA technique

Antigen protein concentration (μg/ml)	ELISA readings at 400 nm Serum E24 1:50		E24 1:500		N10 1:50		N10 1:500	
60.000	0.090	0.110	0.060	0.060	0.030	0.015	0.010	0.010
6.000	0.08	0.105	0.065	0.065	0.035	0.025	0.005	0.015
0.600	0.087	0.087	0.052	0.052	.002	0.000	0.012	0.007
0.060	0.052	0.032	0.042	0.032	.002	.002	0.002	0.002
0.006	0.022	0.032	0.022	0.022	0.012	0.007	0.007	0.002

Readings are corrected for the appropriate saline control reading.
Results in Figure 21 are expressed as the mean of these readings.

Appendix 2c. Figure 22 – Kinetics of the antigen/antibody reaction for the ELISA technique

Time of incubation of Ag/Ab complex (hours)	ELISA readings at 400 nm Serum E24 1:50		E24 1:500		N10 1:50		N10 1:500	
1.5	0.077	0.097	0.027	0.012	0.000	0.002	0.000	0.000
3	0.085	0.085	0.045	0.055	0.025	0.015	0.005	0.005
4.5	0.092	0.097	0.030	0.037	0.002	0.012	0.002	0.002
6	0.137	0.102	0.027	0.052	0.002	0.007	0.002	0.027
7.5	0.140	0.150	0.050	0.085	0.010	0.030	0.010	0.025

Readings are corrected for the saline control reading.
Results in Figure 21 are expressed as the mean of these readings.

Appendix 2d. Figure 23a – Kinetics of the binding of the enzyme conjugate for the ELISA technique

Time of incubation in conjugate	ELISA readings at 400 nm Serum E24 1:50		E24 1:500		N10 1:50		N10 1:500	
1	0.005	0.010	0.000	0.000	0.000	0.000	0.000	0.000
4	0.047	0.062	0.017	0.012	0.000	0.000	0.000	0.000
7	0.112	0.117	0.037	0.042	0.002	0.007	0.002	0.000
24	0.220	0.235	0.085	0.090	0.020	0.015	0.005	0.010

Readings are corrected for the saline control reading.
Results in Table 23a are expressed as the mean of these readings after correction for the appropriate normal serum reading.

Appendix 2e. Figure 23b – Results of a repeat experiment on the kinetics of the enzyme conjugate for the ELISA technique

Time of incubation in conjugate	ELISA readings at 400 nm Serum E23 1:50		E23 1:500		N8 & N9 1:50		N8 &N9 1:500	
15 minutes	0.110	0.110	0.080	0.090	0.030	0.030	0.020	0.020
30 minutes	0.122	0.112	0.092	0.092	0.032	0.042	0.012	0.022
1 hour	0.175	0.185	0.125	0.155	0.067	0.071	0.045	0.055
2 hours	0.215	0.205	0.175	0.165	0.115	0.095	0.085	0.085
4 hours	0.337	0.327	0.267	0.287	0.157	0.197	0.087	0.107
6 hours	0.585	0.625	0.555	0.565	0.235	0.255	0.165	0.175
24 hours	0.740	0.730	0.670	0.690	0.380	0.420	0.270	0.350

Readings are corrected for the appropriate saline control reading
Results in Figure 23b are expressed as the mean of these readings after correction for the appropriate normal serum reading.

Appendix 2f. Figure 24 – Fitting ELISA into a two day schedule

Incubation time in serum (hours)	ELISA readings at 400 nm E24 1:50		N10 1:50		Incubation time in conjugate (hours)
1	0.067	0.092	0.037	0.017	21
2	0.057	0.067	0.000	0.000	20
3	0.075	0.075	0.005	0.005	19
4	0.062	0.107	0.007	0.012	18
5	0.042	0.062	0.012	0.007	17
6	0.052	0.047	0.007	0.007	16

Readings are corrected for the appropriate saline control reading
Results in Figure 24 are expressed as the mean of these readings.

Appendix 2g. Figure 25 – Serum titration for the ELISA technique

Serum dilution	ELISA readings at 400 nm											
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
E21	0.185	0.175	0.175	0.170	0.145	0.075	0.050	0.045	0.005	0.005	0.000	0.000
	0.205	0.180	0.130	0.140	0.135	0.105	0.07	0.025	0.025	0.015	0.000	0.000
E24	0.305	0.240	0.185	0.215	0.175	0.175	0.155	0.130	0.085	0.065	0.115*	0.045
	0.315	0.260	0.165	0.210	0.230	0.220	0.180	0.135	0.105	0.055	0.025	0.015
N10	0.070	0.025	0.035	0.015	0.025	0.025	0.005	0.000	0.000	0.000	0.000	0.000
	0.070	0.040	0.030	0.020	0.020	0.015	0.015	0.005	0.000	0.000	0.000	0.000

*Outlier

Readings are corrected for the saline control reading
Results in Figure 25 are expressed as the mean of these readings.

Appendix 2h. Table 13 – Comparison of methods used to incubate ELISA tubes

Procedure	ELISA readings at 400 nm							
Roller drum	E19 (668)*	E20 (670)	E21 (674)	E22 (67)	E23 (675)	E24 (Z155)	E25	N8 & N9 pool
1	0.063	0.058	0.093	0.123	0.118	0.143	0.043	0.018
2	0.068	0.053	0.088	0.123	0.113	0.153	0.058	0.013
3	0.068	0.063	0.088	0.128	0.123	0.158	0.053	0.008
4	0.073	0.068	0.088	0.188	0.118	0.163	0.063	0.013
5	0.068	0.053	0.098	0.123	0.113	0.173	0.048	0.013
Standing still	E19 (668)	E20 (670)	E21 (674)	E22 (67)	E23 (675)	E25 (Z155)	E25	N8 & N9 pool
1	0.069	0.069	0.089	0.119	0.114	0.149	0.059	0.014
2	0.069	0.074	0.099	0.124	0.109	0.139	0.054	0.000
3	0.069	0.064	0.099	0.119	0.124	0.144	0.049	0.009
4	0.074	0.059	0.089	0.129	0.129	0.149	0.059	0.014
5	0.064	0.049	0.089	0.119	0.119	0.139	0.059	0.009

Readings are corrected for the appropriate saline control reading.
Results in Table 13 are expressed as the mean and standard deviation of these readings.
* Code numbers used by Gallie & Sewell (1976).

Appendix 2i. Table 14 – The standard deviation within the ELISA technique

Serum	ELISA readings at 400 nm																			
E21 1:50	0.239	0.279	0.194	0.209	0.249	0.174	0.204	0.244	0.214	0.179	0.234	0.179	0.209	0.239	0.229	0.164	0.219	0.269	0.104	0.254
E24 1:50	0.269	0.294	0.264	0.194	0.269	0.364	0.364	0.349	0.319	0.344	0.389	0.314	0.319	0.299	0.344	0.399	0.229	0.249	0.349	0.279
E25 1:50	0.189	0.134	0.204	0.134	0.174	0.179	0.204	0.159	0.179	0.184	0.169	0.174	0.139	0.159	0.179	0.189	0.169	0.129	0.089	0.109
N10 1:50	0.034	0.034	0.029	0.039	0.240	0.034	0.024	0.029	0.029	0.029	0.039	0.029	0.029	0.034	0.034	0.029	0.034	0.039	0.034	0.019

Readings are corrected for the saline control reading.
Results in Table 14 are expressed as the mean and standard deviation of these readings.

Appendix 2j. Table 15 – ELISA controls

Control name	ELISA readings at 400 nm		
Positive control/test (E24 1:50)	0.210	0.200	0.280
Antigen control	0.040	–	–
Conjugate control	0.040	–	–
Normal serum control (N10 1:50)	0.010	0.090	–
Antigen control	0.035	–	–
Conjugate control	0.040	–	–
Saline control	0.080	–	–
Antigen saline control	0.035	–	–
Saline conjugate control	0.040	–	–
Antigen substrate control	0.035	–	–
Saline conjugate substrate control	0.040	–	–
Saline substrate control	0.045	–	–

Appendix 2k-a. Tables 16a-d – Comparison of micro and m cro-ELISA for different incubation times and different enzyme conjugates

Serum	ELISA readings at 400 nm							
	E19(668)*	E20(670)*	E21(674)*	E22(67)*	E23(675)*	E24(Z155)*	E25	N8 & N9 pool
Micro ELISA-AP	0.33	0.36	0.48	0.61	0.57	0.73	0.33	0.11
Incubation 4+18 hrs.	0.34	0.37	0.49	0.60	0.56	0.72	0.31	0.17
Read on a 1 cm	1.68**	0.35	0.47	0.59	0.57	0.74	0.32	0.10
light path	0.38	0.28	0.45	0.58	0.53	0.74	0.30	0.15
cuvette	0.35	0.27	0.45	0.57	0.51	0.73	0.33	0.16
Micro ELISA-AP	0.23	0.24	0.34	0.41	0.41	0.40	0.23	0.09
Incubation 30' and 30'	0.23	0.25	0.33	0.41	0.41	0.49	0.21	0.11
at 37°C	0.22	0.24	0.34	0.42	0.40	0.48	0.21	0.09
Read on a 1 cm	0.23	0.25	0.89**	0.41	0.41	0.47	0.21	0.07
light path cuvette	0.21	0.23	0.34	0.40	0.38	0.50	0.22	0.08
Macro ELISA-AP	0.060	0.060	0.100	0.140	0.105	0.135	0.055	0.015
Incubation 4+18 hrs.	0.070	0.040	0.105	0.110	0.115	0.170	0.065	0.020
Read on a 2 mm	0.080	0.050	0.090	0.125	0.120	0.155	0.050	0.050
light path	0.075	0.050	0.100	0.120	0.115	0.210	0.050	0.000
cuvette	0.095	0.055	0.110	0.115	0.120	0.185	0.040	0.000
Macro ELISA-AP	0.060	0.045	0.075	0.085	0.085	0.105	0.045	0.005
Incubation 30' and 30'	0.040	0.050	0.070	0.100	0.090	0.100	0.040	0.000
at 37°C	0.095	0.045	0.075	0.095	0.085	0.140	0.040	0.000
Read on a 2 mm	0.070	0.040	0.080	0.090	0.085	0.130	0.020	0.000
light path cuvette	0.065	0.040	0.085	0.085	0.095	0.140	0.025	0.000

*Code numbers used by Gallie & Sewell (1976)
**Outliers

Readings are corrected for the appropriate saline control reading
Results in Tables 16a-b are expressed as the mean and standard deviation of these readings
Results in Tables 16c-d are expressed as a percentage of the mean standard positive serum reading (E24).

Appendix 2k-b. Tables 16a - d – Comparison of micro and macro-ELISA for different incubation times and different enzyme conjugates

Serum	E19(668)*	E20(670)*	E21(674)*	E22(67)*	E23(675)*	E24(Z155)*	E25	N8 & N9 pool
Micro ELISA-HRPO	0.74	0.52	0.82	1.10	0.94	1.20	0.52	0.14
Incubation 4+18 hrs.	0.66	0.45	0.72	1.05	1.12	1.16	0.44	0.11
Read on 1cm	0.78	0.56	0.64	0.95	1.06	1.32	0.45	0.05
light path	∞**	0.45	0.96	1.10	1.05	1.44	0.46	0.22
cuvette	0.56	0.54	0.62	1.00	0.95	1.50	0.42	0.16
Micro ELISA-HRPO	0.46	0.34	0.53	0.74	0.74	1.02	0.43	0.14
Incubation 30' and 30'	0.39	0.39	0.46	0.84	0.81	1.09	0.32	0.15
at 37°C	0.43	0.39	0.67	0.71	0.88	0.90	0.36	0.04
Read on 1 cm	0.53	0.32	0.60	0.60	0.67	0.88	0.39	0.08
light path cuvette	0.50	0.36	0.53	0.67	0.64	1.16	0.25	0.04
Macro ELISA-HRPO	0.20	0.16	0.23	0.35	0.36	0.47	0.15	0.03
Incubation 4+18 hrs.	0.21	0.17	0.23	0.37	0.32	0.46	0.15	0.10
Read on 2mm	0.19	0.16	0.20	0.37	0.35	0.47	0.16	0.08
light path	0.19	0.15	0.22	0.36	0.36	0.45	0.17	0.08
cuvette	0.19	0.17	0.24	0.34	0.33	0.47	0.16	0.06
Macro ELISA-HRPO	0.16	0.13	0.18	0.27	0.27	0.37	0.14	0.02
Incubation 30' and 30'	0.16	0.14	0.19	0.29	0.27	0.37	0.13	0.06
at 37°C	0.17	0.14	0.18	0.28	0.28	0.35	0.12	0.03
Read on a 2mm	0.17	0.15	0.18	0.29	0.28	0.38	0.12	0.04
light path cuvette	0.16	0.12	0.19	0.26	0.29	0.37	0.13	0.04

*Code numbers used by Gallie & Sewell (1976)
**Outliers

Readings are corrected for the appropriate saline control reading
Results in Tables 16a-b are expressed as the mean and standard deviation of these readings
Results in Tables 16c-d are expressed as a percentage of the mean standard positive serum reading (E24)

Appendix 3a. Table 24 – The ELISA technique using fractions of SE purified by immuno-adsorption

Fraction code numbers (Table 22)	ELISA readings* at 400 nm for various sera									
	E23		E25		PO		Pool N8 & N9		Pool N14 & N15	
SN1	0.030	0.035	0.005	0.005	0.020	0.025	0.015	0.015	0.025	0.025
SN2	0.048	0.068	0.013	0.008	0.028	0.043	0.013	0.003	0.013	0.013
SN3	0.040	0.020	0.010	0.010	0.050	0.050	0.000	0.040	0.050	0.050
SN4	0.030	0.000	0.010	0.010	0.040	0.040	0.045	0.035	0.020	0.030
SN5	0.070	0.025	0.010	0.010	0.070	0.075	0.010	0.005	0.020	0.015
SN6	0.520	0.480	0.060	0.110	0.039	0.420	0.030	0.035	0.085	0.090
SN7	0.120	0.145	0.030	0.020	0.110	0.090	0.010	0.000	0.030	0.020
Untreated SE	0.595	0.580	0.500	0.645	0.590	0.630	0.060	0.070	0.190	0.180

All the ELISA readings were corrected for the appropriate saline control reading.
*ELISA readings corrected to 3 d.p - Antigen protein coated onto ELISA tubes at 25 μg/ml.
Results in Table 24 are expressed as the mean of these readings.

Appendix 3b. Table 25 – The ELISA technique using fractions of SE purified by immunoadsorption

Fraction code numbers (Table 22)	ELISA readings* at 400 nm for various sera									
	E23		E25		PO		Pool N8 &N9		Pool N14 & N15	
SN1	0.010	0.015	0.030	0.010	0.025	0.025	0.000	0.000	0.010	0.000
SN2	0.013	0.003	0.008	0.003	0.018	0.018	0.000	0.000	0.003	0.000
SN3	0.007	0.007	0.002	0.007	0.017	0.022	0.000	0.000	0.007	0.007
SN4	0.040	0.080	0.005	0.001	0.055	0.060	0.060	0.040	0.035	0.030
SN5	0.030	0.025	0.005	0.000	0.035	0.035	0.010	0.000	0.010	0.010
SN6	0.190	0.190	0.035	0.060	0.185	0.200	0.040	0.020	0.030	0.020
SN7	0.085	0.045	0.005	0.010	0.065	0.060	0.025	0.025	0.020	0.015
Untreated SE	0.270	0.315	0.340	0.480	0.490	0.390	0.040	0.030	0.135	0.110

All the ELISA readings were corrected for the appropriate saline control reading.
*ELISA readings corrected to 3 d.p. antigen protein coated onto ELISA tubes at 0.5 μg/ml.
Results in Table 25 are expressed as the mean of these readings.

Appendix 3c. Table 27 – The ELISA inhibition technique using fraction F4 purified by immunoadsorption

Serum	ELISA readings** at 400 nm					
	F4		Unpurified SE		Saline control	
E23(675)*	0.485	0.435	0.130	0.130	0.750	0.750
E24 (Z155)	0.295	0.565	0.190	0.200	0.280	0.390
E25	0.335	0.335	0.110	0.110	0.640	0.640
PO	0.265	0.300	0.080	0.080	0.590	0.600
Pooled N8 & N9	0.015	0.025	0.020	0.020	0.090	0.090
Pooled N14 & N15	0.005	0.000	0.040	0.050	0.180	0.180

*Code numbers used by Gallie & Sewell (1976)
**Corrected to 3 d.p.

These results are corrected for the saline control reading
The results in Table 27 are expresssed as the mean of these readings.

Appendix 3d. Table 28 – The ELISA technique using fraction F4 purified by immunoadsorption

Serum	ELISA readings** at 400 nm			
	F4		Unpurified antigen	
E23(675)*	0.330	0.280	0.850	0.800
E24(2155)	0.400	0.410	0.740	0.760
E25	0.230	0.220	0.600	0.590
PO	0.190	0.220	0.550	0.460
Pooled N8 & N9	0.020	0.030	0.130	0.100
Pooled N14 & N15	0.060	0.040	0.200	0.250

*Code numbers used by Gallie & Sewell (1976)

These results are corrected for the saline control reading
The results in Table 27 are expressed at the mean of these readings.

**Corrected to 3 d.p.

Appendix 4a. Figure 48 – Antigen titration of *T. saginata* larval antigens for use in the ELISA technique

Sample used to coat the ELISA tubes and the antigen/protein content/ml		ELISA-AP readings at 400 nm Serum								
		E24			E25			N10		
SE	6.0 μg/ml	0.059	0.056	0.060	0.240	0.260	0.027	0.030	0.020	0.020
	0.6 μg/ml	0.490	0.500	0.520	0.210	0.210	0.220	0.020	0.020	0.020
	0.06 μg/ml	0.093	0.103	0.103	0.073	0.083	0.078	0.013	0.003	0.013
T. saginata cyst fluid	6.0 μg/ml	0.063	0.053	0.053	0.193	0.223	0.193	0.003	0.003	0.003
	0.6 μg/ml	0.093	0.023	0.023	0.313	0.283	0.343	0.013	0.003	0.003
	0.06 μg/ml	0.017	0.017	0.027	0.217	0.198	0.198	0.007	0.007	0.007
Scolex and wall antigen	6.0 μg/ml	0.550	0.560	0.580	0.340	0.360	0.350	0.060	0.070	0.090
	0.6 μg/ml	0.530	0.480	0.480	0.310	0.380	0.340	0.060	0.070	0.100
	0.06 μg/ml	0.190	0.150	0.160	0.210	0.140	0.120	0.040	0.040	0.020

Readings are corrected for the appropriate saline control reading
Results in Figure 48a are for serum E25 and are expressed as the mean of these readings.
Results in Figure 48b are for serum E24 and are expressed as the mean of these readings.

Appendix 4b. Table 29 – *T. saginata* cyst fluid and SE used as antigens in the ELISA technique

Sample used to coat the ELISA tubes and the antigen/protein content/ml		ELISA-AP readings at 400 nm Serum							
		E19	E20	E21	E25	E22	E23	E24	N10
SE	6.0 μg/ml	0.173	0.103	0.163	0.163	0.263	0.213	0.333	0.003
		0.153	0.083	0.183	0.163	0.253	0.263	0.363	0.003
		0.163	0.103	0.203	0.163	0.293	0.253	0.383	0.003
T. saginata cyst fluid	0.6 μg/ml	0.160	0.150	0.180	0.230	0.060	0.040	0.030	0.000
		0.170	0.140	0.240	0.190	0.060	0.040	0.030	0.000
		0.170	0.180	0.200	0.235	0.070	0.040	0.035	0.000

Readings are corrected for the saline control readings.
Results in Table 29 are expressed as the mean and standard deviation of these readings.

Appendix 4c/i. Figure 49a – Antigen titration for Sephadex G200 reprocessed extracts of SE - Expt A

Sample used to coat ELISA tubes and the antigen protein content/ml		ELISA-AP readings at 400 nm Serum			
		E24		N10	
SE	6.0 μg/ml	0.220	0.270	0.010	0.010
	0.6 μg/ml	0.145	0.165	0.000	0.000
	0.06 μg/ml	0.025	0.035	0.005	0.000
S.1.1.	6.0 μg/ml	0.130	0.130	0.010	0.010
	0.6 μg/ml	0.140	0.150	0.000	0.000
	0.06 μg/ml	0.035	0.045	0.000	0.005
S.2.2.	6.0 μg/ml	0.175	0.205	0.000	0.000
	0.6 μg/ml	0.035	0.045	0.000	0.030
	0.06 μg/ml	0.000	0.000	0.000	0.000

Readings are corrected for the appropriate saline control reading.
Results in Figure 49a are expressed as the mean of these readings.

Appendix 4c/ii. Figure 49b – Antigen titration for Sephadex G200 reprocessed extracts of SE - Expt B

Sample used to coat ELISA tubes and the antigen protein content/ml		ELISA-AP readings at 400 nm Serum			
		E24		N10	
SE	60.0 μg/ml	0.305	0.295	0.025	0.025
	6.0 μg/ml	0.281	0.291	0.026	0.031
	0.6 μg/ml	0.142	0.147	0.017	0.017
	0.06 μg/ml	0.022	0.022	0.007	0.007
S.1.1.	60.0 μg/ml	0.200	0.200	0.025	0.020
	6.0 μg/ml	0.220	0.240	0.015	0.015
	0.6 μg/ml	0.112	0.132	0.012	0.012
	0.06 μg/ml	0.030	0.030	0.010	0.005
S.2.2.	60.0 μg/ml	0.275	0.300	0.015	0.015
	6.0 μg/ml	0.207	0.212	0.017	0.017
	0.6 μg/ml	0.060	0.065	0.010	0.015
	0.06 μg/ml	0.015	0.010	0.015	0.005

Readings are corrected for the appropriate saline control reading.
Results in Figure 49b are expressed as the mean of these readings.

Appendix 4d. Figure 50 – Antigen titration for Sephadex G200 fractionated extracts of SE

Sample used to coat ELISA tubes and the antigen protein content/ml		ELISA-AP readings at 400 nm Serum									
		E24					N10				
SE	6.0 μg/ml	0.335	0.345	0.375	0.390	0.360	0.010	0.015	0.025	0.035	0.015
	0.6 μg/ml	0.210	0.280	0.295	0.290	0.270	0.010	0.010	0.010	0.005	0.005
	0.06 μg/ml	0.065	0.075	0.060	0.060	0.065	0.000	0.005	0.000	0.000	0.000
2.S.1.	6.0 μg/ml	0.309	0.279	0.289	0.289	0.304	0.019	0.004	0.019	0.019	0.019
	0.6 μg/ml	0.236	0.251	0.221	0.231	0.236	0.016	0.011	0.011	0.006	0.011
	0.06 μg/ml	0.067	0.072	0.072	0.077	0.072	0.002	0.002	0.000	0.000	0.002
2.S.2	6.0 μg/ml	0.380	0.400	0.405	0.039	0.415	0.025	0.030	0.030	0.030	0.035
	0.6 μg/ml	0.161	0.151	0.171	0.156	0.176	0.006	0.006	0.006	0.001	0.006
	0.06 μg/ml	0.066	0.061	0.071	0.066	0.061	0.006	0.011	0.011	0.006	0.006
2.S.3.	6.0 μg/ml	0.140	0.135	0.125	0.120	0.125	0.025	0.020	0.025	0.025	0.025
	0.6 μg/ml	0.075	0.007	0.065	0.065	0.060	0.010	0.005	0.010	0.005	0.005
	0.06 μg/ml	0.035	0.035	0.035	0.030	0.035	0.005	0.005	0.010	0.010	0.000

Readings are corrected for the appropriate saline control reading.
Results in Figure 50 are expressed as the mean of these readings.

Appendix 4e. Table 31 – Percentage inhibition obtained in ELISA readings when a sample of serum from a *T. saginata* infected calf was absorbed with sufficient fraction S.1.1. to remove all the haemagglutination activity from the serum

Serum	ELISA-AP readings at 400 nm			
	E24		N10	
Treatment to serum	Non-absorbed	Absorbed	Non-absorbed	Absorbed
Antigen used to coat ELISA tubes at 6.0 μg/ml antigen protein				
SE	0.510	0.208	0.005	0.010
	0.540	0.230	0.010	0.010
	0.550	0.223	0.045	
S.1.1.	0.400	0.145	0.015	0.010
	0.400	0.145	0.025	0.010
	0.510	0.145	0.025	0.010
S.2.2.	0.408	0.190	0.028	0.015
	0.443	0.190	0.013	0.025
	0.443	0.225	0.028	0.015

Readings are corrected for the appropriate saline control reading.
The percentage inhibition was calculated for each of these readings and the results in Table 31 are expressed as the mean and standard deviation of the percentage inhibition.

Appendix 5a. Figure 54 – Serological response of calves to experimental oral infection with *T. saginata* eggs
ELISA-AP readings at 400 nm

Weeks p.i.	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10
E1	- -	- -	- -	- -	0.00 0.00	0.00 0.00	0.00 0.00	0.01 0.00	0.03 0.03	0.10 0.09	0.10 0.12	0.09 0.10	0.10 0.09	0.10 0.10	0.105 0.105	0.09 0.09
E2	0.02 0.02	- -	0.01 0.02	0.01 0.02	0.01 0.02	0.01 0.01	0.01 0.01	0.01 0.02	0.14 0.13	0.125 0.09	0.13 0.13	- -	0.14 0.13	0.19 0.14	0.13 0.16	0.15 0.15
E3	- -	- -	- -	- -	0.02 0.02	0.01 0.02	0.03 0.02	0.03 0.02	0.025 0.02	0.09 0.08	0.11 0.11	0.13 0.13	0.15 0.15	0.15 0.15	- -	0.10 0.13

Weeks p.i.	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
E1	0.09 -	0.9 0.08	0.08 0.08	- -	0.08 0.07	0.08 0.08	0.08 0.06	0.08 0.07	0.08 0.07	0.09 0.07	0.08 0.07	0.08 0.06	0.09 0.09	- -	0.08 0.09	0.10 0.10
E2	0.13 0.13	- -	0.13 0.14	0.15 0.18	0.17 0.15	0.15 0.18	0.15 0.18	0.15 0.18	0.15 0.15	0.20 0.18	0.15 0.17	0.16 0.16	0.15 0.14	0.14 0.14	- -	- -
E3	0.10 0.11	0.11 0.13	0.13 0.13	0.13 0.13	0.125 0.14	0.14 0.14	0.18 0.14	0.17 0.17	0.18 0.19	0.23 0.22	0.21 0.21	0.20 0.21	0.23 0.20	0.19 0.20	0.22 0.24	0.23 0.25

Weeks p.i.	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
E1	0.10 0.11	0.10 0.10	- -	0.10 0.095	0.09 0.10	0.10 0.10	0.09 0.08	0.10 0.09	0.12 0.12	- -	0.10 0.10	- -	0.11 -	0.11 0.13	0.07 0.14	0.12 0.13
E3	0.27 0.25															

Weeks p.i.	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
E1	- -	0.13 0.11	0.13 0.13	0.11 0.12	0.13 0.14	0.14 0.14	0.19 0.15	0.18 0.16	0.19 0.19	0.21 0.21	0.23 0.24	0.24 0.25	0.26 0.27	0.19 0.27	- -	- -

Readings are corrected for saline control readings.
Standard positive serum (E24) .525, .51 mean reading 0.52.
Results in Figure 54 are expressed as a percentage of the standard positive serum reading.

Appendix 5b. Figure 54 – Serological response of calves to experimental oral infection with *T. saginata* eggs
IDH titres

Weeks pi	-5		-4		-3		-2		-1		0		1		2		3		4		5		6		7		8		9		10	
E1	-	-	-	-	-	-	-	-	1:4	*	*	*	*	*	*	*	1:4	1:4	1:4	1:4	1:8	1:8	1:8	1:16	1:8	1:16	1:16	1:16	1:16	1:32	1:16	1:16
E2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	1:4	1:4	1:8	1:8	1:8	1:8	1:8	1:8	-	-	1:8	1:8	1:8	1:16	1:8	1:8	1:16	1:8
E3	-	-	-	-	-	-	-	-	1:4	*	*	*	1:4	1:4	1:8	1:8	1:8	1:16	-	-	1:8	1:16	1:16	1:32	1:16	1:16	1:16	1:16	-	-	1:32	1:32

Weeks pi	11		12		13		14		15		16		17		18		19		20		21		22		23		24		25		26	
E1	1:8	1:16	1:8	1:16	1:8	1:8	-	-	1:8	1:8	1:8	1:16	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:16	1:8	1:8	1:8	1:8	1:16	1:8	-	-	1:16	1:16	1:16	1:16
E2	1:16	1:16	-	-	1:16	1:16	1:16	1:16	1:32	1:16	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:64	1:16	1:32	1:16	1:32	1:16	1:16	1:16	1:32	-	-	-	-
E3	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:16	1:32	1:16	1:32	1:32	1:32	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:8**	1.32	1.64

Weeks pi	27		28		29		30		31		32		33		34		35		36		37		38		39		40		41		42	
E1	1:16	1:16	1:16	1:8	-	-	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	-	-	1:16	1:16	-	-	1:16	1:32	-	-	-	-	1:16	1:32
E3	1:32	1:64																														

Weeks pi	43		44		45		46		47		48		49		50		51		52		53		54		55		56		57		58	
E1	-	-	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:64	1:64	1:64	1:64	1:64	1:64	1:32	1:64	1:32	1:64	1:32	1:64	1:64	1:64	1:32	-	-	-	-

Results in Figure 54 are expressed as a mean of the $\frac{1}{\text{Log}_2}$ value of the IDH titre

**Outlier

*Titre < 1:4

Appendix 6a. Figure 54 – Serological response of calves to experimental oral infection with *T. saginata* eggs
ELISA-AP readings at 400 nm

Weeks pi		-4			-2			0			2			4			6			8			10			12			14			16			18			20		
78*	E6	0.02	0.00	0.025	0.015	0.025	0.00	0.07	0.00	0.09	0.035	0.10	0.05	0.075	0.035	0.085	0.05	0.05	0.04	0.045	0.055	0.08	0.0	0.095	0.025	0.10	0.10
69	E7	0.00	0.00	0.00	0.00	0.00	0.00	0.035	0.00	0.08	0.00	0.08	0.075	0.095	0.06	0.09	0.06	0.085	0.08	0.075	0.06	0.125	0.08	0.14	0.10	0.09	0.09
62	E8	0.00	0.00	0.025	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.005	0.015	0.06	0.00	0.035	0.00	0.05	0.00	0.095	0.00	0.035	0.005	0.075	0.00	0.07	0.01
61	N1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.015	0.015**	0.00	0.00	0.00	0.00	0.00	0.00
64	N2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Weeks pi		22			24			26			28			30			32			34			36			38			40			42			44		
78	E6	0.12	0.045	0.08	0.04	0.12	0.045	0.095	0.075	0.095	0.065	0.085	0.085	0.055	0.085	0.05	0.06	0.035	0.00	0.045	0.05	0.075	0.085	0.025	0.3
69	E7	0.10	0.095	0.11	0.11	0.14	0.145	0.10	0.13	0.13	0.125	0.12	0.125	0.12	0.14	0.115	0.10	0.11	0.09	0.00	0.085	0.085	0.095	0.16	0.125
62	E8	0.02	0.005	0.035	0.00	0.00	0.00	0.00	0.015	0.00	0.00	0.00	0.00	0.00	0.025	0.00	-	0.015	-	0.00	-	0.01	-	0.05	-
61	N1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
64	N2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Readings are corrected for saline control readings
Standard positive serum (E24) O.D. reading at 400 nm 0.115, 0.17, 0.17, 0.21,
Mean standard positive serum reading = 0.17
Results in Figure 54 and 55 are expressed as a percentage of the standard positive serum reading.

*Code numbers used by Gallie & Sewell (1974b)
**Outliers

Appendix 6b. Figure 54 – Serological response of calves to experimental oral infection with *T. saginata* eggs
IDH titres

Weeks pi		-4		-2		0		2		4		6		8		10		12		14		16		18		20	
78**	E6	*	*	*	*	*	*	*	*	*	*	1:16	1:8	1:64	1:16	1:64	1:32	1:64	1:64	1:64	1:32	1:64	1:32	1:64	1:32	1:128	1:64
69	E7	*	*	*	*	*	*	*	1:4	*	*	1:32	1:64	1:64	1:64	1:128	1:64	1:128	1:128	1:256	1:128	1:128	1:512	1:512	1:512	1:512	1:1024
62	E8	*	*	*	*	*	*	1:4	1:4	1:4	*	1:4	1:4	1:8	1:8	1:16	1:16	1:16	1:16	1:16	1:8	1:16	1:16	1:16	1:16	1:16	1:16
61	N1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
64	N2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Weeks pi		22		24		26		28		30		32		34		36		38		40		42		44	
78	E6	1:128	1:64	1:128	1:128	1:256	1:128	1:256	1:64	1:128	1:64	1:64	1:64	1:32	1:64	1:32	1:32	1:16	1:16	1:16	1:16	1:8	1:16	1:16	1:32
69	E7	1:512	1:2048	1:512	1:1024	1:512	1:512	1:1024	1:512	1:512	1:256	1:512	1:256	1:512	1:256	1:512	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256
62	E8	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:32	1:8	1:32	1:16	1:32	1:16	1:32	1:16	1:16	1:16	1:16	1:16	1:32
61	N1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
64	N2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Results in Figure 54 are expressed as a mean of the $\frac{1}{\text{Log}_2}$ value of the IDH titre.

*Titre ∠1:4

Results in Figure 55 expressed as a percentage of mean $\frac{1}{\text{Log}_2}$ value of the IDH titre for E24 (1:1024, 1:2048) = 10.5.

**Code numbers used by Gallie & Sewell (1974b)

Appendix 7a. Figures 27a and b – Serological response of a calf to experimental oral infection with *T. saginata* eggs and Figure 56 ELISA-AP readings at 400 nm

Weeks pi		-4			-3			-2			-1			0			1			2		
78*	E6	0.015	0.015	0.015	0.020	0.020	0.025	0.020	0.015	0.020	0.010	0.010	0.015	0.015	0.010	0.015	0.020	0.020	0.015	0.015	0.020	0.020
61	N1	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-		-

		3			4			5			6			7			8		
78*	E6	0.060	0.060	0.055	0.080	0.080	0.095	0.095	0.105	0.115	0.115	0.120	0.115	0.125	0.125	0.125	0.125	0.085	0.110
61	N1	-		-	0.005	0.010	0.010	0.010	0.010	0.005	0.010	0.010	0.005	0.010	0.010	0.010	0.005	0.005	0.005

Weeks pi		9			10			11			12			13			14			15		
78*	E6	0.145	0.130	0.140	-		-	0.145	0.130	0.125	0.145	0.140	0.145	0.145	0.135	0.130	0.145	0.125	0.145	0.145	0.145	0.125
61*	N1	0.005	0.005	0.000	0.005	0.005	0.005	0.005	0.000	0.000	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005

		19			23			27			31			35			37		
78*	E6	0.135	0.135	0.120	0.145	0.170	0.150	0.160	0.180	0.175	0.160	0.170	0.145	0.110	0.135	0.110	0.100	0.100	0.090
61*	N1	0.005	0.000	0.005	0.010	0.010	0.010	0.015	0.010	0.010	0.010	0.010	0.005	0.010	0.010	0.005	0.005	0.010	0.005

Readings are corrected for saline control reading.
Results on Figure 27a are expressed as ELISA readings at 400nm.
Results on Figure 27b are expressed as a percentage of standard positive serum (E24)
Results on Figure 56 are expressed as a percentage of standard positive serum (E25)
SE was used as the antigen to coat the ELISA tubes.

ELISA readings at 400 nm = 0.325, 0.370, 0.380 $\bar{X}$ = 0.358.
ELISA readings at 400 nm = 0.130, 0.145, 0.130 $\bar{X}$ = 0.135.
*Code numbers used by Gallie & Sewell (1974b).

Appendix 7b. Figure 56 – Serological response of a calf to experimental oral infection with *T. saginata* eggs
ELISA-AP readings at 400 nm

Weeks pi		-4		0		4		8		12		16		20		24		28		32		36	
Group 1																							
78*	E6	0.005	0.015	0.020	0.020	0.050	0.060	0.060	0.065	0.080	0.060	0.070	0.050	0.075	0.055	0.075	0.090	0.085	0.100	0.095	0.080	0.080	0.085
61*	N1	0.010	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.005	0.000	0.005	0.000	0.005	0.010	0.005	0.005	0.010	0.000	0.010	0.005	0.005	0.010
Group 2																							
78*	E6	0.000	0.005	0.000	0.000	0.030	0.025	0.045	0.035	0.030	0.035	0.015	0.015	0.05	0.055	0.065	0.030	0.035	0.040	0.070	0.010	0.040	0.050
61*	N1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Readings are corrected for the appropriate saline control reading
Results in Figure 56 are expressed as a percentage of the standard positive serum E25
Sephadex G200 fraction S.1.1. of SE was used as antigen to coat the ELISA tubes for Group 1
Sephadex G200 fraction S.2.2. of SE was used as antigen to coat the ELISA tubes for Group 2

*Code numbers used by Gallie & Sewell (1974b)

Antigen	ELISA-AP readings at 400 nm for standard positive serum E25
S.1.1.	0.130, 0.120 $\bar{X}$ = 0.125
S.2.2.	0.045, 0.05 $\bar{X}$ = 0.048

Appendix 7c. Figure 56 – Serological response of a calf to experimental oral infection with *T. saginata* eggs
ELISA-AP readings at 400 nm

Weeks pi		-4			-3			-2			-1			0			1			2			3		
78*	E6	0.005	0.005	0.000	0.005	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.005	0.000	0.005	0.010	0.005	0.005	0.005	0.010	0.005	0.005	0.010	0.010	0.015
61	N1	0.005	0.000	0.005	0.000	0.000	0.000	0.005	0.010	0.005	0.000	0.005	0.005	0.000	0.005	0.010	0.005	0.000	0.005	0.025	0.040	0.030	0.025	0.025	0.025

		4			5			6			7			8			9			10		
78	E6	0.015	0.015	0.025	0.030	0.015	0.015	0.015	0.020	0.015	0.025	0.020	0.020	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
61	N1	0.010	0.015	0.010	0.005	0.005	0.005	0.005	0.010	0.005	0.005	0.005	0.005	0.005	0.005	0.010	0.010	0.010	0.010	0.005	0.010	0.010

Weeks pi		11			12			13			14			15			16			17			18		
78	E6	0.030	0.025	0.030	0.030	0.020	0.030	0.035	0.025	0.030	0.025	0.025	0.025	0.025	0.030	0.030	0.030	0.025	0.030	0.040	0.030	0.025	0.030	0.035	0.035
61	N1	0.010	0.030	0.040	0.015	0.010	0.000	0.010	0.010	0.010	0.010	0.010	0.015	0.045	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000

		19			20			21			22			23			24			25		
78	E6	0.045	0.030	0.040	0.040	-	0.045	0.040	0.030	0.030	0.035	0.045	0.045	0.040	0.045	0.040	-	-	-	0.045	0.035	0.030
61	N1	0.000	0.000	0.000	0.005	0.005	0.010	0.005	0.005	0.005	0.005	0.010	0.005	0.005	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.015

Appendix 7c continued

Weeks pi		26			27			28			29			30			31			32			33		
78	E6	0.030	0.035	0.035	0.035	0.040	0.045	-	-	-	0.035	0.050	0.045	0.040	0.045	0.045	0.040	0.035	0.040	0.035	0.050	0.035	0.030	0.030	0.040
61	N1	0.010	0.015	0.015	0.020	0.020	0.020	0.020	0.025	0.025	0.010	0.010	0.015	0.015	0.015	0.020	0.015	0.010	0.015	0.010	0.010	0.010	0.010	0.010	0.015

		34			35			36			37			–			–			–					
78	E6	0.030	0.030	0.030	0.025	0.030	0.035	0.025	0.030	0.020	0.025	0.015	0.025		–			–			–				
61	N1	0.015	0.010	0.020	0.010	0.015	0.015	0.020	0.015	0.015	0.020	0.020	0.015		–			–			–				

Readings are corrected for the appropriate saline control reading
Results in Figure 56 are expressed as the percentage of the mean standard positive serum (E25) reading
Sephadex G200 fraction S.2.2. of SE was used as antigen to coat the ELISA tubes

ELISA-AP readings at 400 nm (E25) = 0.045, 0.025, 0.030 $\bar{X}$ = 0.033
*Code numbers used by Gallie & Sewell (1974b)

Appendix 8. Figure 57 – Serological response of calves orally infected with *T. saginata* eggs to i/m injection with SE
ELISA-AP readings at 400 nm

Weeks pi	-6		-5		-4		-3		-2		-1		0		1		2		3		4		5		6		7		8	
Group 1 Control																														
(E1)																														
Expt A	0.69	0.70	-	-	0.77	0.62	-	-	0.72	0.72	-	-	0.56	0.64	0.48	0.58	0.53	0.55	-	-	0.47	0.47	-	-	0.45	0.47	-	-	0.38	0.36
Expt B	0.41	0.42	-	-	-	-	-	-	0.38	0.33	-	-	0.34	0.325	0.28	0.31	0.29	0.33	0.20	0.28	0.19	0.29	0.19	0.27	0.13	0.30	0.16	0.28	0.14	0.26
(E2)																														
Expt A	0.31	0.41	-	-	0.22	0.31	-	-	0.27	0.33	-	-	0.25	0.33	0.50	0.63	0.74	0.81	-	-	0.69	0.70	-	-	0.60	0.68	-	-	0.79	0.80
Expt B	-	-	-	-	-	-	-	-	0.37	0.24	-	-	0.21	0.26	0.315	0.34	0.38	0.42	0.37	0.42	0.37	0.43	0.38	0.38	0.35	0.39	0.37	0.39	0.40	0.41
(E3)																														
Expt A	0.56	0.60	-	-	0.54	0.52	-	-	0.48	0.60	-	-	0.46	0.64	0.64	0.62	0.74	0.64	-	-	0.68	0.68	-	-	0.62	0.69	-	-	1.09	0.87
Expt B	-	-	-	-	-	-	-	-	0.25	0.31	-	-	0.34	0.31	0.24	0.31	0.24	0.26	0.31	0.34	0.30	0.35	0.30	0.35	0.33	0.37	0.30	0.32	0.42	0.46

Weeks pi	-6		-5		-4		-3		-2		-1		0		1		2		3		4		5		6		7		8	
Group 2 Control																														
(E1)																														
Expt A	0.36	0.37	-	-	0.40	0.43	-	-	0.28	0.33	-	-	0.37	0.29	0.37	0.33	0.29	0.36	-	-	0.33	0.30	-	-	0.19	0.29	-	-	0.27	0.25
Expt B	0.145	0.105	-	-	-	-	-	-	0.095	0.275	-	-	0.125	0.235	0.105	0.155	0.125	0.005	0.105	0.185	0.125	0.055	0.100	0.145	0.195	0.145	0.125	0.095	0.155	0.155
(E2)																														
Expt A	0.21	0.31	-	-	0.31	0.17	-	-	0.36	0.26	-	-	0.25	0.29	0.35	0.37	0.34	0.44	-	-	0.36	0.46	-	-	0.31	0.39	-	-	0.39	0.38
Expt B	-	-	-	-	-	-	-	-	0.145	0.175	-	-	0.115	0.145	0.135	0.115	0.125	0.095	0.065	0.105	0.105	0.105	0.045	0.065	0.075	0.025	0.075	0.055	0.085	0.085
(E3)																														
Expt A	0.47	0.43	-	-	0.40	0.38	-	-	0.38	0.31	-	-	0.35	0.36	0.36	0.42	0.35	0.27	-	-	0.29	0.36	-	-	0.38	0.37	-	-	0.39	0.34
Expt B	-	-	-	-	-	-	-	-	0.065	0.135	-	-	0.075	0.115	0.105	0.095	0.135	0.065	0.075	0.095	0.085	0.095	0.085	0.125	0.145	0.095	0.155	0.015	0.125	0.105

Group 1 Saline extract of *T. saginata* used as antigen in the ELISA technique
Group 2 *T. saginata* cysticercal fluid used as antigen in the ELISA technique
Readings are corrected for the saline control reading.
Results in Figure 57 are expressed as a percentage of the standard positive serum E25.
Day 0 was 45 weeks after the original oral infection for calf E1.
Day 0 was 33 weeks after the original oral infection for calf E2.
Day 0 was 36 weeks after the original oral infection for calf E3.

ELISA results for the standard positive serum (E25)

	Coating antigen	ELISA-AP readings at 400 nm		Mean reading
Expt A	saline extract	0.50	0.47	0.485
	cyst fluid	0.40	0.44	0.42
Expt B	saline extract	0.32	0.37	0.345
	cyst fluid	0.275	0.215	0.245

Appendix 9. Tables 33a and b – Preliminary ELISA results for two groups of calves neonatally infected with *T. saginata* eggs and a group of controls.
ELISA-AP readings at 400 nm

	SERIAL DOSE GROUP														
	E9-(P36)*			E10(C14)			E11(409)			E12(423)			E13(C98)		
Expt A Week 33	0.083	0.078	0.083	0.088	0.098	0.098	0.068	0.078	0.083	0.053	0.043	0.048	0.043	0.043	0.023
Week 34	0.078	0.088	0.093	0.093	0.083	0.098	0.068	0.068	0.068	0.033	0.033	0.028	0.043	0.033	0.048
Expt B Week 33	0.050	0.050	0.050	0.090	0.100	0.090	0.040	0.055	0.055	0.020	0.020	0.010	0.030	0.030	0.030
Week 34	0.060	0.060	0.080	0.085	0.100	0.110	0.060	0.060	0.050	0.010	0.010	0.010	0.035	0.040	0.050

	SINGLE DOSE GROUP														
	E14(411)			E15(418)			E16(420)			E17(L14)			E18(A1)		
Expt A Week 33	0.053	0.058	0.048	0.033	0.023	0.028	0.033	0.048	0.038	0.063	0.083	0.133	0.033	0.028	0.033
Week 34	0.068	0.048	0.058	0.028	0.023	0.028	0.048	0.048	0.033	0.078	0.108	0.103	0.028	0.008	0.008
Expt B Week 33	0.050	0.050	0.050	0.010	0.000	0.005	0.040	0.040	0.050	0.000	0.000	0.000	0.010	0.010	0.010
Week 34	0.050	0.060	0.020	0.000	0.020	0.005	0.090	0.090	0.070	0.000	0.000	0.000	0.010	0.010	0.010

	CONTROL GROUP														
	N3(V60)			N4(80)			N5(79)			N6(V57)			N7(83)		
Expt A Week 33	0.028	0.028	0.028	0.028	0.018	0.023	0.028	0.023	0.028	0.023	0.013	0.018	0.028	0.038	0.023
Week 34	0.023	0.023	0.018	0.028	0.023	0.023	0.028	0.033	0.033	0.028	0.023	0.028	0.028	0.028	0.028
Expt B Week 33	0.000	0.000	0.000	0.010	0.010	0.010	0.010	0.010	0.010	0.005	0.000	0.000	0.010	0.010	0.010
Week 34	0.005	0.000	0.000	0.005	0.000	0.000	0.005	0.005	0.005	0.000	0.000	0.000	0.020	0.010	0.005

Expt A (Table 33b) SE was used as antigen in the ELISA technique
Expt B (Table 33a) *T. saginata* cysticercal fluid was used as antigen in the ELISA technique
The readings are corrected for the appropriate saline control reading.

*Codes used by Gallie & Sewell (1974a).

Appendix 10 - Figure 58 - Serological response of neonate calves to oral infection with *T. saginata* eggs (ELISA-AP readings at 400 nm)

Months p.i.	O	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Serially dosed group															
(P36)* E9	0.51,0.32	0.79,0.75	0.37,0.39	0.41,0.54	0.55,0.57	0.47,0.50	0.65,0.58	0.53,0.53	0.67,0.73	0.91,0.91	0.69,0.73	0.74,0.79	1.09,1.13	0.83,0.69	0.57,0.55
(C14) E10	0.29,0.28	0.51,0.43	0.3,0.28	0.47,0.28	0.39,0.33	0.45,0.53	0.55,0.51	0.53,0.51	0.51,0.49	0.73,0.63	0.65,0.63	0.62,0.67	0.65,0.65	0.61,0.63	0.45,0.43
(409) E11	0.29,0.25	0.23,0.19	0.19,0.23	0.35,0.35	0.59,0.61	0.55,0.63	0.67,0.77	1.07,1.11	0.97,1.15	0.65,0.53	0.43,0.37	0.33,0.29	0.27,0.27	0.29,0.31	0.15,0.17
(423) E12	0.32,0.33	0.31,0.39	0.17,0.17	0.15,0.19	0.15,0.22	0.25,0.25	0.21,0.23	0.21,0.21	0.23,0.23	0.23,0.22	0.19,0.23	0.25,0.25	0.37,0.35	0.31,0.27	0.19,0.15
(C98) E13	0.15,0.13	0.69,0.65	0.19,0.21	0.27,0.25	0.27,0.29	0.43,0.41	0.28,0.27	0.62,0.57	0.37,0.4	0.53,0.55	0.65,0.63	0.51,0.5	0.44,0.43	0.75,0.81	0.23,0.17
Singly dosed group															
(411)* E14	0.15,0.17	0.52,0.67	0.19,0.19	0.37,0.37	0.15,0.29	0.49,0.45	0.49,0.41	0.49,0.41	0.43,0.41	0.37,0.33	0.37,0.40	0.51,0.57	0.51,0.58	0.57,0.49	0.13,0.11
(418) E15	0.41,0.33	0.23,0.31	0.30,0.29	0.25,0.21	0.25,0.25	0.14,0.11	0.39,0.31	0.23,0.22	0.23,0.22	0.20,0.17	0.19,0.21	0.51,0.22	0.22,0.27	0.27,0.27	0.34,0.27
(420) E16	0.12,0.17	0.94,1.02	0.20,0.20	0.34,0.31	0.40,0.21	0.54,0.61	0.35,0.55	0.44,0.43	0.35,0.35	0.63,0.42	1.01,1.23	0.83,0.91	0.77,0.77	0.67,0.71	0.19,0.19
(L14) E17	0.43,0.41	0.64,0.57	0.27,0.25	0.29,0.30	0.80,0.92	0.67,0.67	0.77,0.94	0.97,0.99	1.24,1.27	1.17,1.20	1.05,1.27	1.23,1.23	1.05,1.11	0.97,1.00	0.43,0.39
(A1) E18	0.57,0.47	0.72,0.73	0.28,0.29	0.25,0.25	0.27,0.29	0.27,0.26	0.31,0.29	0.32,0.23	0.27,0.23	0.33,0.27	0.27,0.32	0.28,0.30	0.32,0.27	0.27,0.33	0.37,0.39
Controls															
(V60)* N3	0.15,0.17	0.22,0.31	0.27,0.28	0.19,0.17	0.17,0.17	0.10,0.10	0.13,0.11	0.13,0.17	0.17,0.15	0.23,0.17	0.17,0.23	0.19,0.19	0.23,0.23	0.23,0.20	0.27,0.31
(80) N4	0.23,0.17	0.27,0.29	0.13,0.11	0.13,0.09	0.17,0.13	0.17,0.17	0.69**,0.37**	0.19,0.14	0.19,0.17	0.15,0.14	0.23,0.25	0.31,0.19	0.23,0.15	0.22,0.27	0.23,0.25
(79) N5	0.40,0.51	0.53,0.65	0.09,0.19	0.11,0.15	0.13,0.15	0.11,0.16	0.15,0.15	0.18,0.17	0.13,0.14	0.17,0.15	0.17,0.27	0.17,0.18	0.13,0.22	0.21,0.23	1.02,1.12
(V57) N6	0.40,0.37	0.33,0.29	0.34,0.41	0.29,0.31	0.18,0.21	0.17,0.17	0.17,0.17	0.20,0.20	0.17,0.18	0.17,0.17	0.23,0.21	0.15,0.17	0.17,0.15	0.21,0.13	0.29,0.33
(83) N7	0.27,0.31	0.34,0.41	– –	– –	0.14,0.13	0.21,0.21	0.19,0.25	0.15,0.17	0.19,0.13	0.19,0.22	0.26,0.21	0.23,0.25	0.25,0.27	0.24,0.36	0.29,0.31

Readings are corrected for saline control readings
Standard positive serum (E24) diluted 1:2 ELISA-AP readings at 400 nm 1.22,1.11= 2.44,2.22 mean standard reading = 2.33
Results in Figure 58 are expressed as a percentage of the standard positive serum reading
Untreated SE was used as antigen and the results read using a 1 cm light path cuvette instead of the normal 2 mm cuvette.

*Code numbers used by Gallie & Sewell (1974a)
** Outliers

Appendix 11. Figure 59 – Serological response of calves given hatched and activated *T. saginata* eggs intramuscularly
ELISA-AP readings at 400 nm

Weeks post injection	-1		0		1		2		3		4		5		6	
Expt. 1																
Expt. A E4	0.00	0.07	0.09	0.09	0.07	0.06	0.08	0.10	-	-	0.12	0.012	0.18	0.14	0.21	0.19
Expt. B E4	0.05	0.05	0.06	0.03	0.05	0.04	0.07	0.05	-	-	0.08	0.06	0.08	0.11	0.115	0.11
Expt. A E5	0.06	0.04	0.02	0.01	0.025	0.02	0.04	0.04	-	-	0.12	0.11	0.10	0.10	0.27	0.23
Expt. B E5	0.00	0.02	0.045	0.03	0.03	0.04	0.04	0.03	-	-	0.06	0.07	0.05	0.06	0.17	0.18

	7		8		9		10		11		12		13	
Expt. A E4	0.22	0.19	0.21	0.16	0.19	0.018	0.14	0.15	0.14	0.16	0.165	0.15	0.11	0.12
Expt. B E4	0.12	0.10	0.08	0.14	0.08	0.08	0.10	0.105	0.08	0.11	0.07	0.09	0.06	0.09
Expt. A. E5	0.27	0.265	0.23	0.20	0.26	0.20	0.11	0.17	0.21	0.07	0.19	0.15	0.135	0.09
Expt. B E5	0.14	0.15	0.12	0.13	0.10	0.12	0.10	0.08	0.07	0.14	0.13	0.15	0.15	0.11

Appendix 11 continued

Weeks post injection	-1		0		1		2		3		4		5		6	
Expt. 2																
Expt. A E4	0.03	0.10	0.08	0.01	0.12	0.16	0.02	0.01	-	-	0.09	0.06	0.07	0.02	0.05	0.04
Expt. B E4	0.035	0.025	0.00	0.00	0.005	0.005	0.00	0.00	-	-	0.015	0.00	0.00	0.00	0.75	0.00
Expt. A E5	0.02	0.07	0.02	0.07	0.00	0.01	0.03	0.05	-	-	0.04	0.01	0.10	0.05	0.10	0.13
Expt. B E5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	0.00	0.00	0.005	0.00	0.00	0.00

	7		8		9		10		11		12		13	
Expt. A E4	0.01	0.00	0.00	0.01	0.08	0.12	0.04	0.07	0.06	0.05	0.04	0.04	0.07	0.04
Expt. B E4	0.00	0.025	0.00	0.00	0.045	0.00	0.055	0.00	0.025	0.00	0.00	0.00	0.00	0.00
Expt. A E5	0.12	0.11	0.07	0.09	0.08	0.08	0.06	0.11	0.05	0.09	0.13	0.07	0.08	0.07
Expt. B E5	0.025	0.005	0.015	0.005	0.015	0.00	0.045	0.075	0.055	0.045	0.035	0.075	0.055	0.065

Readings are corrected for saline control readings
Standard positive serum (E25)
Results in Figure 59 are expressed as a percentage of the standard positive serum reading

Expt. A – SE used as antigen in the ELISA technique
Expt. B – *T. saginata* cysticercal fluid used as antigen in the ELISA technique

ELISA results for the standard positive serum (E25)

	Coating antigen	ELISA reading at 400 nm		Mean reading
Expt. 1	SE	0.50	0.47	0.485
	cyst fluid	0.40	0.44	0.42
Expt. 2	SE	0.32	0.37	0.345
	cyst fluid	0.275	0.215	0.245

Appendix 12. Table 34 – Comparative study of sera from experimentally infected cattle from Scotland, Germany and Hungary. (ELISA-AP readings at 400 nm)

Weeks p.i.		−2	−1	0	1	2	3	4	5	6	7	8
Scottish calves	E6	0.025 0.015	–	0.025 0.000	–	0.070 0.000	–	0.090 0.030	–	0.100 0.050	–	0.075 0.035
	E7	0.000 0.000	–	0.000 0.000	–	0.035 0.000	–	0.080 0.000	–	0.080 0.075	–	0.085 0.060
	E8	0.025 0.000	–	0.030 0.000	–	0.000 0.000	–	0.000 0.000	–	0.005 0.015	–	0.060 0.000
German calves	E26	–	0.035 0.035	–	–	–	0.025 0.02	–	–	–	–	–
	E27	–	0.005 0.005	–	–	–	0.075 0.005	–	–	–	–	–
	E28	–	–	–	–	–	–	–	–	–	–	–
	E29	–	–	–	–	–	–	–	–	–	–	–
Hungarian calves	E30	–	–	–	–	–	–	–	–	–	0.125 0.165	0.105 0.085
	E31	–	–	–	–	–	–	–	–	–	0.075 0.065	0.095 0.085

Weeks p.i.		9	10	11	12	13	14	15	16	42	60	–
Scottish calves	E6	–	0.085 0.050	–	0.050 0.040	–	0.045 0.055	–	0.080 0.030	0.075 0.085	–	–
	E7	–	0.090 0.060	–	0.085 0.080	–	0.075 0.060	–	0.125 0.080	0.085 0.095	–	–
	E8	–	0.035 0.000	–	0.050 0.000	–	0.095 0.000	–	0.035 0.005	0.010 –	–	–
German calves	E26	–	–	–	–	–	–	0.195 0.195	–	–	0.105 0.095	–
	E27	–	–	–	–	–	–	0.135 0.175	–	–	–	–
	E28	–	–	–	0.125 0.145	–	–	–	–	–	–	–
	E29	–	–	–	–	–	–	–	0.125 0.145	–	–	–
Hungarian calves	E30	0.125 0.115	–	–	–	–	0.105 0.105	–	–	–	–	–
	E31	0.100 0.095	–	–	–	–	0.090 0.085	–	–	–	–	–

Results are corrected for saline control reading
Results are expressed in Table 34 as a percentage of the standard positive serum (E24)
Untreated SE was used as antigen

Standard positive serum			E24			$\bar{X}$
Experiment A Scottish calves (Appendix 6)	0.115	0.170	0.170	0.210		0.170
Experiment B German and Hungarian calves	0.325		0.285			0.305

Appendix 13a. Tables 35-37 – Total protein, albumin and globulin levels in the sera of cattle on farms A, B and C.

Farm A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Total protein g/100ml	6.4	6.8	5.8	5.9	5.8	5.7	6.6	6.6	5.9	6.7	6.2	6.2
Globulin g/100ml	3.49	4.22	2.90	3.10	3.31	3.65	3.73	3.37	3.13	3.89	3.44	3.35
Albumin g/100ml	2.91	2.58	2.90	2.80	2.49	2.05	2.87	3.23	2.77	2.81	2.76	2.85
	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24
Total protein g/100ml	5.9	5.9	6.1	6.1	5.8	6.0	6.0	5.8	6.2	6.2	6.4	6.5
Globulin g/100ml	3.54	3.07	3.02	3.20	3.02	3.54	3.57	4.39	3.60	3.35	3.71	4.19
Albumin g/100ml	2.36	2.83	3.08	2.90	2.78	2.46	2.43	2.35	2.60	2.85	2.69	2.24

Farm B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14
Total protein g/100ml	7.6	6.1	6.3	5.8	6.5	6.3	6.6	6.8	5.9	6.3	7.7	6.3	5.9	6.4
Globulin g/100ml	4.10	3.02	3.34	2.87	3.25	3.18	3.04	3.30	3.01	3.40	4.20	3.37	2.80	2.85
Albumin g/100ml	3.50	3.08	2.96	2.93	3.25	3.12	3.56	3.50	2.89	2.90	3.50	2.93	3.10	3.55

Farm C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
Total protein g/100ml	7.5	6.8	7.3	6.6	7.0	7.3	7.3	6.8	6.9	6.7	6.2
Globulin g/100ml	4.65	4.08	4.56	4.16	4.89	4.09	4.49	4.15	4.31	4.05	3.97
Albumin g/100ml	2.85	2.72	2.74	2.44	3.12	3.21	2.81	2.65	2.59	2.65	2.23

Total protein corrected to 1 dp. globulin and albumin to 2 dp.

Appendix 13b. Table 38 - IDH results for sera of cattle on farms A, B and C.

Farm A	A1		A2		A3		A4		A5		A6		A7		A8		A9		A10		A11		A12	
Expt. A	1:8	1:8	*	*	1:16	1:16	1:8	1:8	1:8	1:8	1:32	1:32	1:8	1:8	1:32	1:32	1:8	1:8	1:8	1:8	*	*	1:8	1:8
Expt. B	1:4	1:4	1:8	1:8	*	*	*	*	1:4	1:4	1:8	1:8	1:32	1:32	1:8	1:8	1:32	1:32	1:4	1:4	*	*	1:4	1:4
Expt. C	1:16	1:16	1:32	1:32	1:16	1:16	1:32	1:16	1:32	1:32	1:32	1:32	1:256	1:256	1:64	1:64	1:64	1:128	1:16	1:16	1:4	1:4	1:8	1:16

	A13		A14		A15		A16		A17		A18		A19		A20		A21		A22		A23		A24	
Expt. A	1:4	1:4	1:16	1:16	1:4	1:4	1:8	1:8	1:8	1:8	*	*	1:4	1:4	1:8	1:8	1:16	1:16	*	*	1:8	1:8	1:16	1:16
Expt. B	1:4	1:4	1:16	1:16	1:4	1:4	1:4	1:4	1:4	1:4	*	*	*	*	1:4	1:4	1:8	1:8	*	*	1:8	1:8	1:8	1:8
Expt. C	1:32	1:32	1:32	1:32	1:8	1:8	1:8	1:8	1:16	1:16	*	*	1:4	1:4	1:16	1:16	1:32	1:32	1:4	1:4	1:16	1:16	1:16	1:16

Farm B	B1			B2			B3			B4			B5			B6			B7		
Expt. A	1:8		1:8	1:16		1:16	1:8		1:8	1:64		1:64	1:32		1:32	1:4		1:4	1:4		1:4
Expt. B	1:4	1:4	1:16	1:4	1:4	1:8	1:4	1:4	1:8	1:16	1:16	1:64	1:16	1:16	1:64	1:4	1:4	1:8	1:4	1:4	1:4
Expt. C	1:8		1:16	1:4		1:4	1:4		1:8	1:32		1:16	1:32		1:16	*		1:4	1:4		1:4

	B8			B9			B10			B11			B12			B13			B14		
Expt. A	1:32		1:16	1:8		1:8	1:16		1:16	1:64		1:64	1:16		1:16	1:64		1:64	1:32		1:32
Expt. B	1:8	1:8	1:16	1:4	1:4	1:8	1:4	1:4	1:16	1:16	1:16	1:64	1:4	1:4	1:32	1:16	1:16	1:128	1:8	1:8	1:32
Expt. C	1:8		1:8	1:4		1:4	1:8		1:4	1:32		1:16	1:8		1:16	1:16		1:16	1:4		1:16

Farm C	C1		C2		C3		C4		C5		C6		C7		C8		C9		C10		C11	
Expt. A	*	*	*	*	1:4	1:4	*	*	1:8	1:8	*	*	1:8	1:8	1:4	1:4	1:4	1:4	*	*	1:4	1:4
Expt. B	*	*	*	*	*	*	*	*	1:4	1:4	*	*	1:8	1:8	*	*	1:4	1:4	*	*	*	*
Expt. C	*	*	1:4	*	*	*	*	*	1:4	1:4	*	*	1:4	1:4	1:4	1:4	1:4	1:4	*	*	*	*

The IDH titre is expressed as the last dilution of serum to show any haemagglutination
Results for Table 38 are expressed as a Log_{10} of the reciprocal of the titre
Untreated SE was used as antigen

Appendix 13c. Table 39 - SAFA results from sera of cattle on farms A, B and C.
(SAFA readings at an excitation wavelength of 465 nm)

Farm A	A1		A2		A3		A4		A5		A6		A7		A8		A9		A10		A11		A12	
SAFA values	0.060	0.040	0.070	0.040	0.010	0.000	0.030	0.030	0.020	0.020	0.010	0.040	0.040	0.060	0.060	0.040	0.030	0.035	0.035	0.020	0.020	0.020	0.020	

	A13		A14		A15		A16		A17		A18		A19		A20		A21		A22		A23		A24	
SAFA values	0.010	0.000	0.030	0.020	0.040	0.030	0.020	0.020	0.010	0.010	0.010	0.000	0.010	0.000	0.020	0.010	0.060	0.050	0.010	0.000	0.030	0.020	0.010	0.000

	E24	
SAFA values	0.110	0.120

Farm B	B1		B2		B3		B4		B5		B6		B7		B8		B9		B10		B11		B12	
SAFA values	0.035	0.025	0.025	0.025	0.035	0.005	0.025	0.015	0.065	0.035	0.045	0.025	0.045	0.000	0.025	0.025	0.025	0.025	0.075	0.045	0.065	0.055	0.045	0.075

	B13		B14		E24	
SAFA values	0.000	0.015	0.055	0.065	0.155	0.185

Farm C	C1		C2		C3		C4		C5		C6		C7		C8		C9		C10		C11		E24	
SAFA values	0.020	0.010	0.000	0.000	0.000	0.010	0.030	0.030	0.010	0.000	0.010	0.020	0.020	0.010	0.000	0.010	0.000	0.010	0.010	0.000	0.000	0.000	0.100	0.070
	0.030	0.020	0.000	0.000	0.020	0.020	0.010	0.020	0.000	0.000	0.030	0.020	0.030	0.030	0.000	0.000	0.000	0.020	0.020	0.000	0.000	0.010	0.080	0.060

Readings are corrected for the normal bovine serum reading.
Results for Table 39 are compared as a percentage of the mean standard positive serum reading (E24)
Untreated SE was used as antigen.

Appendix 13d. Tables 40-45 – ELISA results from sera of cattle on farms A, B and C. (ELISA-AP readings at 400 nm)

Farm A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Untreated												
SE	0.007,0.001	0.015,0.000	0.000,0.000	0.000,0.007	0.005,0.007	0.017,0.007	0.003,0.007	0.017,0.007	0.025,0.029	0.075,0.000	0.001,0.015	0.027,0.033
S1.1	0.048,0.048	0.026,0.022	0.028,0.032	0.020,0.026	0.014,0.018	0.020,0.020	0.022,0.024	0.020,0.020	0.022,0.020	0.022,0.028	0.014,0.018	0.018,0.008
S2.2	0.003,0.003	0.007,0.005	0.019,0.009	0.009,0.025	0.017,0.019	0.027,0.025	0.019,0.035	0.015,0.013	0.007,0.005	0.011,0.017	0.017,0.000	0.023,0.011

	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24	E24
Untreated													
SE	0.015,0.029	0.053,0.033	0.027,0.043	0.000,0.003	0.000,0.013	0.015,0.000	0.019,0.013	0.039,0.009	0.005,0.025	0.007,0.005	0.023,0.071	0.003,0.023	0.107,0.103
S1.1	0.012,0.010	0.016,0.020	0.024,0.010	0.010,0.016	0.023,0.018	0.022,0.020	0.026,0.036	0.028,0.032	0.044,0.068	0.022,0.028	0.038,0.030	0.028,0.012	0.088,0.094
S2.2	0.025,0.031	0.013,0.059	0.029,0.029	0.017,0.019	0.009,0.029	0.043,0.037	0.029,0.025	0.035,0.025	0.069,0.025	0.029,0.007	0.043,0.045	0.023,0.033	0.073,0.085

Farm B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13
Untreated													
SE	0.180,0.200	0.120,0.120	0.100,0.100	1.080,1.160	0.600,0.500	0.140,0.100	0.100,0.100	0.120,0.120	0.080,0.060	0.100,0.120	0.100,0.060	0.100,0.100	0.160,0.280
S1.1	0.060,0.080	0.100.0.150	0.120,0.110	0.870,1.070	0.370,0 250	0.080,0.080	0.110,0.070	0.130,0.130	0.080,0.070	0.110,0.080	0.070,0.060	0.080,0.080	0.060,0.100
S2.2	0.130,0.110	0.190,0.170	0.150,0.150	1.350,1.370	0.750,0.590	0.150,0.150	0.070,0.050	0.130,0.090	0.090,0.090	0.250,0.250	0.250,0.190	0.190,0.250	0.390,0.410

	B14	E24
Untreated		
SE	0.130,0.100	1.340,1.405
S1.1	0.080,0.030	0.980,0.960
S2.2	0.070,0.070	0.950,1.050

* Outliers

Farm C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	E24
Untreated												
SE	0.100,0.090	0.120,0.120	0.140,0.140	0.140,0.140	0.100,0.120	0.220,0.240	0.100,0.060	0.100,0.120	0.220,0.240	0.120,0.090	0.120,0.100	1.340,1.405
S1.1	0.060,0.050	0.030,0.060	0.110,0.080	0.090,0.100	0.080,0.050	0.210,0.160	0.100,0.060	0.030,0.060	0.100,0.100	0.040,0.030	0.080,0.060	0.980,0.960
S2.2	0.190,0.270	0.300,0.290	0.270,0.350	0.290,0.230	0.170,0.250	0.190,0.170	0.090,0.190	0.130,0.110	0.150,0.110	0.030,0.150	0.170,0.110	0.950,1.050

Readings are corrected for the saline control readings
Standard positive serum (E24)
Results in Table 40-45 are compared as a percentage of the mean standard positive serum reading (E24)

Appendix 14a. Figure 60 and Table 46 – Serological results for sera from 5 Dutch cattle

	Weeks	IDH titres 0		2		4		6		8		10	
D1	Expt. A	1:16	1:16	1:16	1:8	1:4	1:4	1:8	1:8	1:4	1:4	1:16	1:16
	Expt. B	1:8	1:8	1:8	1:8	*	*	1:16	1:16	1:8	1:4	1:16	1:16
D2	Expt. A	1:8	1:8	1:8	1:8	1:8	1:8	1:4	1:4	1:8	1:8	1:8	1:8
	Expt. B	1:8	1:8	1:16	1:8	1:8	1:8	1:4	1:4	1:8	1:8	1:8	1:8
D3	Expt. A	1:16	1:8	1:8	1:8	1:8	1:4	1:4	1:4	1:8	1:8	1:8	1:8
	Expt. B	1:16	1:16	1:8	1:8	1:4	1:4	1:8	1:8	1:8	*	1:8	1:8
D4	Expt. A	1:8	1:8	1:8	1:8	1:32	1:32	1:4	1:4	1:4	1:4	1:4	1:8
	Expt. B	1:8	1:8	1:8	1:8	1:16	1:16	1:4	1:4	1:8	1:8	1:8	1:8
D5	Expt. A	1:16	1:8	1:4	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:16
	Expt. B	1:8	1:8	1:4	1:4	1:4	1:4	1:8	1:8	1:8	1:8	1:8	1:8

Results in Figure 60 and Table 46 are expressed as the mean of the $\frac{1}{\text{Log}_{10}}$ value of the IDH titre.

Appendix 14b. Figure 60 and Table 46 – Serological results for sera from 5 Dutch cattle

Weeks	ELISA readings at 400 nm 0		2		4		6		8		10	
D1	0.125	0.145	0.125	0.145	0.095	0.075	0.095	0.095	0.095	0.055	0.135	0.105
D2	0.145	0.165	0.125	0.145	0.205	0.215	0.125	0.125	0.155	0.145	0.085	0.075
D3	0.075	0.085	0.095	0.085	0.100	0.125	0.085	0.085	0.085	0.085	0.125	0.135
D4	0.095	0.095	0.065	0.065	0.095	0.135	0.085	0.085	0.085	0.095	0.095	0.095
D5	0.145	0.135	0.125	0.135	0.105	0.085	0.115	0.105	0.145	0.125	0.135	0.115

E24 ELISA readings at 400 nm

0.955, 0.995 $\bar{X} = 0.975$

Readings are corrected for the saline control reading.
Results in Figure 60 are expressed as a percentage of a standard positive serum (E24)
Results in Table 46 are expressed as the mean and standard deviation of the ELISA values.

CORRIGENDUM

for polysaccaride read polysaccharide